REMARKS

This Response provides further evidence that the present invention has patentable utility and is enabled. This evidence could not have been submitted earlier, because the Examiner's basis for asserting a lack of utility has changed from a lack of a credible utility in the Final Office Action, to a lack of specific or substantial utility in the Advisory Action. (See Examiner's Interview Summary record dated July 28, 2005)

Thus, the present Response and literature evidence submitted herewith should be considered at this time.

THE PRESENT INVENTION

Claim 1, which is the only claim pending in the application, reads as follows:

> 1. A congenic rat comprising a mutant GPR10 gene, wherein said congenic rat is obtained by crossing a Otsuka Long-Evans Tokushima Fatty (OTELF) rat (ATCC No. 72016) with a wild-type rat, and wherein said congenic rat exhibits a prolonged immobilization time in a forced swim test compared to said wild-type rat and a prolonged time spent in open arms in an elevated plus-maze test compared to said wild-type rat, and wherein said mutant GPR10 gene contains a G to A substitution at the third position of the coding region.

As described, for example, at pages 3 and 28-33 of the specification, the claimed congenic rat displays depression and anti-anxiety-like behavior, and is thus useful for assessing drug candidates for anti-depressant and anxiolytic activity.

CLAIM REJECTIONS UNDER 35 USC §§ 101 AND 112

At page 2 of the Office Action, the Examiner rejects claim 1 under 35 U.S.C. §101, as lacking patentable utility, and under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement. The Examiner believes that mutations in GPR10 are manifested as different phenotypes in humans, mice and rats, and thus the claimed congenic rat cannot credibly serve as a model of depression or anxiety.

Applicants responded to the rejection on June 23, 2005, establishing to the Examiner's satisfaction that the claimed congenic rat has a credible utility.

An Advisory Action issued on July 15, 2005, stating:

> [T]he claims have credible utility, but lack substantial or specific utility.... Further, the claim lacks enablement as there is no evidence that the rat GPR10 is associated with depression.

A personal interview was held on July 28, 2005, during which the Examiner clarified that claim 1 has credible utility, but that specific and substantial utility should be further demonstrated by a showing that the phenotype of the claimed congenic rat correlates to depression or anxiety. More specifically, the Examiner requested that Applicants demonstrate

that the tests disclosed in the specification are known in the art to correlate to depression and/or anxiety.

In addition, the Examiner clarified that the utility and enablement rejections are coextensive, that is, if claim 1 has patentable utility then claim 1 is also enabled.

FURTHER RESPONSE TO THE UTILITY AND ENABLEMENT REJECTIONS

The following literature evidence is submitted herewith to establish that the claimed congenic rat, which exhibits a prolonged immobilization time in a forced swim test compared to a wild-type rat and a prolonged time spent in open arms in an elevated plus-maze test compared to a wild-type rat, has a specific and substantial utility.

(1) Porsolt et al., *Nature* 266:730 (1977).

(2) Porsolt et al., *European J. Pharmacol.* 47:379-391 (1978).

(3) Hawkins et al., *Nature* 274:512 (1978).

(4) Cannizzaro et al., *Brain Research*, 953: 170-180 (2002).

(5) Mombereau et al., *Neuropsychopharmacology* 29: 1050-1062 (2004).

(6) U.S. Patent 6,936,607

(7) Pellow et al., *J. Neurosci. Methods* 149-167 (1985).

(8) Dawson et al., *Psychopharmacology* 121:109-117 (1995).

Porsolt et al. (1977), in introducing the forced swim test, states that the test meets an important problem in the art:

> A major problem in the search for new antidepressant drugs is the lack of animal models which both resemble depressive illness and are selectively

> sensitive to clinically effective antidepressant treatments. We have been working on a new behavioural model in the rat which attempts to meet these two requirements.

Porsolt et al. (1978) teach:

> [S]everal aspects of our findings suggest that the immobility observed in water is a relatively specific depressive phenomenon. Firstly, it is apparent that the effects observed with the different antidepressant treatments were not merely due to a stimulation of motor activity. Indeed, antidepressant effects as measured by reductions in immobility in the water generally occurred at doses which otherwise decreased activity as measured in the open field. (See Porsolt et al. at pages 386 and 387)
>
> It is noteworthy that the present procedure, which is based on behavioural rather than biochemical concepts, is the first animal model which clearly predicts an antidepressant action for mianserin. (See Porsolt et al. page 388)

Hawkins et al. (1978), extols the Porsolt forced swim test: "We believe that the rat swimming test of Porsolt et al. is a valuable contribution to drug screening methodology and we are now using it in our present work."

Cannizzaro et al. (2002) teach (at the paragraph bridging the left and right columns of page 177) that when rats are less stressed, they display a decreased escape-oriented activity in the forced swim test (FST). Further, acute GABA/BDZ R agonist (DZ) treatment, at doses that decrease anxiety-related behavior, increase immobility time in the FST.

Mombereau et al. (2004) teach that $GABA_B$ -/- mice showed more anxious behavior than wild-type littermates in the light-dark box test, and that these mice also showed decreased immobility (antidepressant-like behavior) in the forced swim test. See Abstract of Mombereau. Further, at page 1058, Mombereau states:

> The mouse forced swim test is the most widely used experimental paradigm for detecting antidepressant activity and to assess alterations in depression-like behavior in genetically modified animals.

U.S. Patent 6,936,607 teaches at column 3:

> The Forced Swim Test (FST) is a behavioral test that is used to screen compounds for antidepressant efficacy. This test is widely used, relatively easy to perform, and sensitive to the effects of some of the major classes of antidepressant drugs, including TCAs and MAOIs, and various atypical antidepressants. Furthermore, this test is relatively selective for antidepressant drugs in the sense that few psychoactive drugs produce similar behavioral actions in the FST.

Pellow et al. (1985) in introducing the elevated +-maze test, state:

> A novel test for the selective identification of anxiolytic and anxiogenic drug effects in the rat is described, using an elevated +-maze consisting of two open arms and two closed arms. The use of this test for detecting such drug effects was validated behaviourally, physiologically, and pharmacologically. (see Abstract)

Dawson et al. (1995) employs the rat elevated plus-maze test to study the anxiolytic activity of a drug compound.

CONCLUSION

The inescapable conclusion from the above literature evidence is that the Forced Swim Test and elevated plus-maze test are well known in the art for assessing depression-like and anxious-like behavior in rats.

Thus, the claimed congenic rat which exhibits a prolonged immobilization time in a forced swim test compared to a wild-type rat, and a prolonged time spent in open arms in an elevated plus-maze test compared to a wild-type rat, clearly has a specific, substantial and credible utility of screening anti-depressant and/or anxiolytic drug candidates.

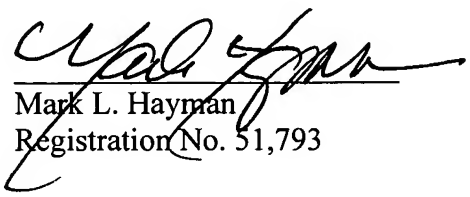
Further, since the section 101 and section 112 rejections are coextensive (see Examiner's interview summary), the claimed congenic rat is also enabled.

In view of the above, withdrawal of the rejections are respectfully requested.

Reconsideration and allowance of this application are now believed to be in order, and such actions are hereby solicited. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

Respectfully submitted,



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Date: September 30, 2005

	180						185							190					195					
Factor XI	Val	Cys	Ala	Gly	Tyr	Arg	-	Glu	Gly	Gly	Lys	-	-	Asp	Ala	Cys	Lys	Gly	Asp	SER	Gly	Gly	Pro
Factor XII*	Leu	Cys	Ala	Gly	Phe	Leu	-	Glu	Gly	Gly	Thr	-	-	Asp	Ala	Cys	Gln	Gly	Asp	SER	Gly	Gly	Pro
Factor IX_a†	Phe	Cys	Ala	Gly	Tyr	His	-	Glu	Gly	Gly	Lys	-	-	Asp	Ser	Cys	Gln	Gly	Asp	SER	Gly	Gly	Pro
Factor X_a†‡	Phe	Cys	Ala	Gly	Tyr	Asp	-	Thr	Gln	Pro	Glu	-	-	Asp	Ala	Cys	Gln	Gly	Asp	SER	Gly	Gly	Pro
Thrombin§	Phe	Cys	Ala	Gly	Tyr	Lys	Pro	Gly	Glu	Gly	Lys	Arg	Gly	Asp	Ala	Cys	Glu	Gly	Asp	SER	Gly	Gly	Pro

Fig. 2 Active site sequence of bovine factor XI and several other blood coagulation proteins. Amino-terminal sequence analysis was performed on 1.2-mg samples of peak 6, Fig. 1, with a Beckman sequencer Model 890A. The operation of this instrument and the methods used are adaptations[16] of Edman and Begg's technique[17]. Phenylthiohydantoin amino acids were identified by gas chromatography after silylation or directly by high-pressure liquid chromatography[18]. Amino acid residues in factor XI that are identical with other plasma serine proteases are shown in blocks. —, spaces inserted to bring the five proteins into alignment for better homology. The numbering system is that of chymotrypsin where the active site serine is residue 195. * Ref. 19; † ref. 20; ‡ ref. 21; § ref. 22.

homogeneous as measured by sodium dodecyl sulphate polyacrylamide gel electrophoresis and immunoelectrophoresis[5]. It has a molecular weight of 124,000 as determined by sedimentation equilibrium and is composed of two similar or identical polypeptide chains held together by a disulphide bond(s). We present here data showing that bovine factor XI contains an amino acid sequence which is very similar to the active site region of many other serine proteases involved in the coagulation process.

S-pyridylethyl factor XI was digested with cyanogen bromide and the polypeptide fragments were fractionated by gel filtration on Sephadex G-75 (Fig. 1). Twelve ultraviolet-absorbing peaks were obtained. Peak 6 (shown by the arrow) migrated as a single band on sodium dodecyl sulphate polyacrylamide gel electrophoresis and had a molecular weight of approximately 5,500. The amino-terminal sequence of this polypeptide chain is shown in Fig. 2, with the active site regions of several other coagulation proteins from bovine plasma. Twenty residues were identified in this peptide. The amino-terminal residue was valine and no other amino acids were detected. The active site serine, corresponding to serine 195 in chymotrypsin, appeared in position 17 of the peptide. The yield of this residue, however, was poor. For the remaining amino acids, the repetitive yields for the degradations were about 95% and over 80% of the expected quantities of the phenylthiohydantoins based on the weight of the lyophilised peptide. It is clear from these data that bovine factor XI contains an amino acid sequence which is characteristic of the active site regions of a number of plasma serine proteases. This sequence is also homologous to that found in pancreatic trypsin[6] and is consistent with the fact that factor XI_a has esterase activity towards benzoylarginyl ethyl ester[3] and participates in blood coagulation by splitting an arginyl valyl peptide bond in factor IX (P. Lindquist, K. Fujikawa and E. W. Davie, in preparation). A specificity toward basic amino acids is also consistent with the fact that residue 189 in factor XI is aspartic acid. This residue is in the bottom of the binding pocket in trypsin and forms an ion pair with an arginine or lysine residue in the substrate[7–13].

Bovine factor XI, like factor XII (Hageman factor), factor IX_a, factor X_a (activated Stuart factor) and thrombin, also contains an aspartic acid residue in position 194 next to serine 195. When trypsinogen and chymotrypsinogen are converted to enzymes, aspartic acid 194 forms an ion pair with the newly formed α-amino group of isoleucine which is generated during the activation reaction[13,14]. It seems likely that a similar mechanism may occur when factor XI is converted to a serine protease by limited proteolysis.

Factor XI contains 12 methionine residues per 124,000 g of glycoprotein[5]. This should yield 14 cyanogen bromide peptides for a molecule composed of two non-identical chains, or seven peptides for a molecule composed of two identical chains. Approximately 12 cyanogen bromide fractions were detected by gel filtration on Sephadex G-75 (Fig. 1). Examination of each of these peaks by sodium dodecyl sulphate polyacrylamide gel electrophoresis indicated, however, that many of these peaks were not homogeneous. Thus, the question as to whether factor XI is composed of two identical chains or two very similar chains remains to be answered.

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Depression: a new animal model sensitive to antidepressant treatments

A MAJOR problem in the search for new antidepressant drugs is the lack of animal models which both resemble depressive illness and are selectively sensitive to clinically effective antidepressant treatments. We have been working on a new behavioural model in the rat which attempts to meet these two requirements. The method is based on the observation that a rat, when forced to swim in a situation from which there is no escape, will, after an initial period of vigorous activity, eventually cease to move altogether making only those movements necessary to keep its head above water. We think that this characteristic and readily identifiable behavioural immobility indicates a state of despair in which the rat has learned that escape

Table 1 Effects of various psychotropic drugs on the total duration of immobility induced in rats forced to swim in a narrow cylinder for 5 min

Experimental treatment	Dose (mg kg^{-1} i.p.)	No. of rats per group	Duration of immobility (s) (mean±s.e.)	P (difference from control)
Tricyclic antidepressants				
Imipramine HCl	Control	5	224.4±4.7	—
	7.5	5	188.2±14.6	N.S
	15	5	138.0±14.5	< 0.01
	30	5	132.4±25.9	< 0.01
Desimipramine HCl	Control	5	214.2±8.2	—
	2.5	5	220.8±22.8	NS
	5	5	202.2±14.3	NS
	10	5	174.8±16.8	NS
	20	5	98.8±17.6	< 0.01
Amitriptyline HCl	Control	10	245.4±8.0	—
	3.75	5	184.4±5.5	< 0.05
	7.5	10	189.7±18.9	< 0.05
	15.0	10	150.7±22.2	< 0.01
Monoamine oxidase inhibitor				
Nialamide HCl	Control	10	224.1±9.6	—
	40	10	231.2±20.3	NS
	80	5	127.4±29.6	NS
	100	5	83.4±43.0	< 0.01
ECS	Control	10	238.7±13.2	—
	ECS	10	166.0±24.7	< 0.05
Atypical antidepressants				
Iprindole HCl	Control	10	243.1±14.1	—
	7.5	5	268.4±11.1	NS
	15	5	237.8±15.9	NS
	30	5	229.4±21.3	NS
	40	5	155.6±24.8	< 0.01
	60	5	260.6±15.8	NS
Mianserin HCl	Control	5	210.6±21.1	—
	3.75	5	187.2±8.9	NS
	7.5	5	187.6±11.2	NS
	15	5	140.0±14.7	< 0.01
	30	5	147.2±11.5	< 0.05
	60	5	256.4±16.6	NS
Viloxazine HCl	Control	10	214.8±12.1	—
	7.5	5	207.6±30.2	NS
	12.5	5	205.6±12.9	NS
	15	5	202.6±21.1	NS
	25	5	176.0±23.2	NS
	30	5	171.0±15.1	NS
	50	5	144.8±15.3	< 0.05
Psychostimulants				
(+)-Amphetamine sulphate	Control	5	202.8±17.9	—
	0.75	5	175.0±9.2	NS
	1.5	5	85.4±19.4	< 0.01
	3.0	5	25.4±23.2	< 0.01
Caffeine	Control	5	219.6±20.7	—
	3.75	5	257.4±8.7	NS
	7.5	5	177.2±21.7	NS
	15	5	99.8±21.0	< 0.01
Anxiolytics				
Chlordiazepoxide HCl	Control	5	237.6±8.9	—
	2	5	237.2±16.0	NS
	4	5	225.6±9.6	NS
	8	5	234.6±9.4	NS
Diazepam	Control	5	208.0±10.7	—
	0.5	5	224.6±10.7	NS
	1	5	242.6±12.7	NS
	2	5	216.6±19.8	NS
	4	5	222.2±21.1	NS
Major tranquillisers				
Chlorpromazine HCl	Control	5	218.2±2.2	—
	0.75	5	220.6±11.8	NS
	1.5	5	224.0±18.1	NS
	3	5	263.0±8.2	< 0.05
Ro4-1284	Control	5	202.6±7.3	—
	1	5	271.2±12.6	< 0.01
	2	5	271.0±12.0	< 0.01
	4	5	257.8±15.9	< 0.01

The rats had previously received three intraperitoneal injections 24, 5 and 1 h before testing. The doses, expressed in terms of the salt, are those given at each injection. The drugs were dissolved or suspended in 0.9% NaCl and injected in a constant volume of 5 ml kg^{-1}. Differences from control values were assessed for statistical significance using the Dunnett test (two-tailed)[18].

is impossible and resigns itself to the experimental conditions. This hypothesis receives support from results presented below which indicate that immobility is reduced by different treatments known to be therapeutic in depression including three drugs, iprindole, mianserin and viloxazine which although clinically active[1-3] show little or no 'antidepressant' activity in the usual animal tests[4-6].

Naive male Sprague–Dawley (Charles River) rats weighing between 160 and 180 g are plunged individually into a vertical plexiglass cylinder (height 40 cm; diameter 18 cm) containing 15 cm of water maintained at 25 °C. After 15 min in the cylinder they are removed and allowed to dry for 15 min in a heated enclosure (32 °C) before being returned to their individual cages. This treatment produces long periods of immobility in the water (10–12 min total duration) and the rats on removal are mildly hypothermic (−3 °C) and are hypoactive for periods up to 30 min. 24 h later the rats are replaced in the cylinder and the total duration of immobility is measured during a 5-min test. Rats submitted to this procedure will remain immobile for 75% of the duration of the test (see Table 1) thus providing a suitable baseline for measuring the effects of drugs which decrease or even increase immobility.

Drugs and doses investigated are shown in Table 1. To optimise the pharmacological effect[7] and at the same time to imitate more closely clinical usage, we administered the drugs in a series of three intraperitoneal injections 24, 5 and 1 h before testing on day 2, the first injection being given immediately before replacing the animals in their cages on day 1. Electroconvulsive shock (ECS; 30 mA, 50 Hz, 1 s) was delivered through ear-clip electrodes according to the same time schedule as for drug injections.

Table 1 shows that all the antidepressants tested as well as ECS significantly reduced immobility. With the tricyclic compounds, particularly amitriptyline, this 'antidepressive' effect was accompanied by lowered muscle tonus and diminished motor activity outside the test situation suggesting sedation. Biphasic effects were observed with mianserin and iprindole: a reduction in immobility at lower doses followed at higher doses by a return to control levels and a marked loss of muscle tonus. Viloxazine decreased immobility without apparent sedative effects. The monoamine oxidase inhibitor, nialamide, reduced immobility but induced small repetitive body movements which were not observed with the tricyclic antidepressants; loss of muscle tonus was noted at 100 mg kg^{-1}. The two psychostimulants (+)-amphetamine and caffeine also reduced immobility but in contrast to the tricyclic compounds induced a marked hyperactivity; with (+)-amphetamine stereotyped head movements were observed at 1.5 and 3 mg kg^{-1}. Neither chlordiazepoxide nor diazepam affected the duration of immobility even at doses which produced noticeable ataxia. In contrast, both chlorpromazine and the reserpine-like compound RO4-1284 increased mobility; with RO4-1284 a ceiling effect was already observed at 1 mg kg^{-1}, a non-cataleptic dose.

We conclude from the results obtained that the immobility induced in these experiments reflects a state of lowered mood in the rat. Immobility was reduced by antidepressant drugs and ECS, was unaffected by anxiolytics, and was increased by drugs known to be capable of inducing depressive states in man[8,9]. The positive findings obtained with amphetamine and caffeine do not stand in contradiction to this conclusion as both compounds do possess some clinical antidepressant activity[10] and besides, the effects observed could be distinguished qualitatively from those of the non-stimulant antidepressants.

The results obtained with mianserin deserve comment because this compound, although clinically active[2], possesses a profile of pharmacological and biochemical activity which is almost the mirror image of that expected of a classical tricyclic antidepressant: it potentiates the effects of reserpine and antagonises the effects of amphetamine[11]. It does not inhibit the re-uptake of monoamines[12] and is moreover a potent blocker of central 5-hydroxytryptamine receptors[13]. Although providing no explanation for the mechanisms of action of mianserin, it is noteworthy that the present procedure, which is based on behavioural rather than biochemical concepts, is the first animal test model which would predict an antidepressant action for this compound. Furthermore the bimodal effect observed strikingly parallels clinical observations showing an optimal antidepressant effect at intermediate doses[14].

These positive findings, together with those obtained with the two other atypical compounds, iprindole and viloxazine, raise the intriguing possibility that the method described here might be capable of discovering new types of antidepressant agents hitherto undetectable using classical screening tests.

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Benzodiazepine receptors in rat brain

HIGH affinity binding of tritium labelled morphine and morphine-like drugs to membranes in brain homogenates[1-3] was a decisive advance in the characterisation of opiate receptors and the discovery of enkephalines and endorphines. We report here experiments which suggest that another important group of psychoactive drugs, the benzodiazepines, bind to specific receptors on the membranes of rat brain cells. This suggests that there may be an unknown endogenous neurotransmitter which is the natural ligand for the benzodiazepine receptor. The binding sites are distributed unevenly through the brain, and displacement potencies of benzodiazepines correlate with pharmacological effects predictive of anxiolytic activity.

Whole forebrains (excluding cerebellum and pons-medulla) of 150 g Wistar rats were homogenised gently in 20 volumes of ice-cold 0.32 M sucrose, centrifuged at 1,000*g* for 10 min and recentrifuged at 30,000*g* to give a crude P_2-synaptosomal fraction. The P_2-fraction was rehomogenised in an equal volume of hypotonic, 50 mM, Tris-HCl (*p*H 7.5). The binding assay consisted of 500 µl test drug solution and with ^{3}H-diazepam as the radioactive ligand, usually at 1.6 nM (^{3}H-diazepam (*N*-methyl-^{3}H) 14, 4 Ci mmol^{-1}; provided by Dr Willy Haefely, Hoffmann-La Roche). The mixture was preincubated for 5 min at 37 °C before addition of ^{3}H-diazepam followed by a 15 min additional incubation. The

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BEHAVIOURAL DESPAIR IN RATS: A NEW MODEL SENSITIVE TO ANTIDEPRESSANT TREATMENTS

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Rats when forced to swim in a cylinder from which they cannot escape will, after an initial period of vigorous activity, adopt a characteristic immobile posture which can be readily identified. Immobility was reduced by various clinically effective antidepressant drugs at doses which otherwise decreased spontaneous motor activity in an open field. Antidepressants could thus be distinguished from psychostimulants which decreased immobility at doses which increased general activity. Anxiolytic compounds did not affect immobility whereas major tranquilisers enhanced it. Immobility was also reduced by electroconvulsive shock, REM sleep deprivation and "enrichment" of the environment. It was concluded that immobility reflects a state of lowered mood in the rat which is selectively sensitive to antidepressant treatments. Positive findings with atypical antidepressant drugs such as iprindole and mianserin suggest that the method may be capable of discovering new antidepressants hitherto undetectable with classical pharmacological tests.

Tricyclic antidepressants Mianserin Depression MAOI ECS
Behavioural model

1. Introduction

A major problem in the search for new psychotropic drugs is the absence of specific animal models for the different mental disease states. This is particularly true in the case of depression, where available screening methods are based mainly on empirically established relationships between the clinical efficacy of known antidepressants and their effects on various pharmacological test models. Because no direct relationship exists between most pharmacological tests for antidepressants and depressive illness itself, it seems unlikely that such empirically based methods will lead to the discovery of antidepressant agents with modes of action different from those already in use. The inadequacy of presently available methods for finding novel antidepressants is demonstrated by the recent discovery of several drugs, for example iprindole and mianserin, which although clinically effective, show little or no "antidepressant" activity in the usual animal tests. While there do exist several convincing behavioural models of depression in experimental animals (Scott et al., 1973; Harlow and Suomi, 1974; Seligman, 1975) none has so far been used for the routine screening of drugs probably for reasons of cost or practicability. There is thus an urgent need for new and simple tests which bear a closer relationship to the clinical phenomena of depression and are selectively sensitive to treatments known to be effective in depressive illness.

We have recently described a behavioural screening test which attempts to meet these requirements (Porsolt et al., 1977). The test is based on the observation that rats when forced to swim in a restricted space from which they cannot escape will eventually cease apparent attempts to escape and

become immobile apart from the small movements necessary to keep their heads above water. We suggested that this characteristic and readily identifiable behavioural immobility reflects a state of despair in the rat and showed that immobility was reduced by a variety of agents which are therapeutically effective in depression.

The experiments reported below expand these original findings and show that reduction of immobility by antidepressant treatments can be clearly dissociated from mere stimulant effects on locomotor activity. Further findings indicate that the depressive behaviour measured in this test is also alleviated by non-pharmacological treatments such as electroconvulsive shock, deprivation of REM sleep and exposure to an "enriched" environment.

2. Materials and methods

2.1. Animals

Male Sprague—Dawley (Charles River) rats weighing 160—180 g were used. They were brought into the laboratory at least one day preceding an experiment and were housed singly in macrolon cages (24 × 11 × 8 cm) with free access to food and water.

2.2. Induction and measurement of immobility

Naive rats were individually forced to swim inside vertical plexiglass cylinders (height: 40 cm; diameter: 18 cm) containing 15 cm of water maintained at 25°C. After 15 min in the water they were removed and allowed to dry for 15 min in a heated enclosure (32°C) before being returned to their home cages. They were replaced in the cylinder 24 h later and the total duration of immobility was measured during a 5 min test. The rat was judged to be immobile whenever it remained floating passively in the water in a slightly hunched but upright position, its head just above the surface (fig. 1).

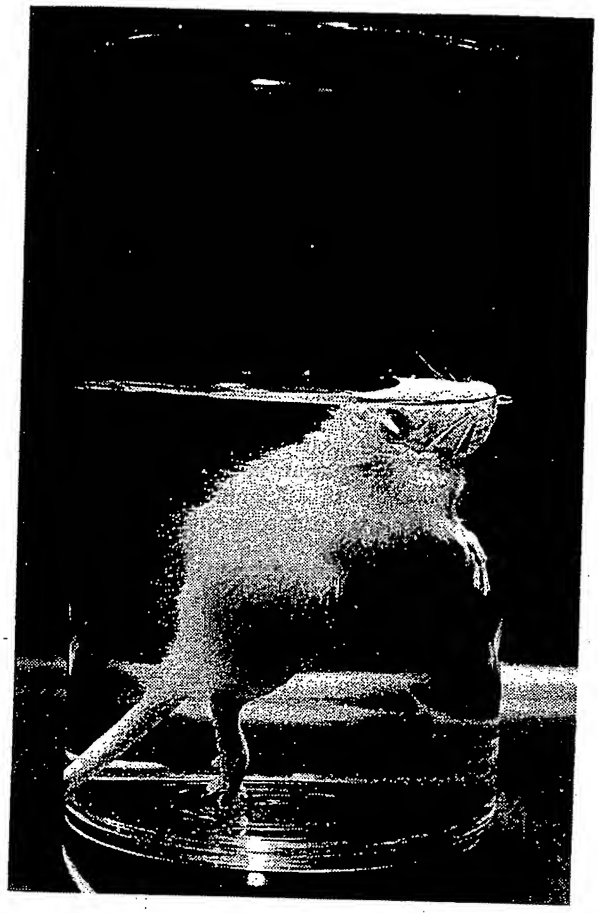

Fig. 1. Rat showing typical posture of immobility after 10 min immersion in water.

2.3. Measurement of locomotor activity

Naive rats were placed individually in one corner of an open field apparatus similar to that described by Soubrié (1971) and the number of quadrants entered in 5 min was counted.

2.4. Drug treatment

The drugs and doses tested are shown in table 3. The drugs were either dissolved in distilled water or dispersed in a suspension of Tween 80 (0.2% w/v 0.9% NaCl). Control animals were given the vehicle only. On the basis of preliminary experiments with imipramine (Results, section 3.2.) an injection schedule

was chosen in which drugs were administered as a series of 3 i.p. injections 24, 5 and 1 h before the 5 min test on the second day. The first injection was given at the end of the drying period (i.e. 15 min after removal from the water). Drugs were injected in a constant volume of 0.5 ml/kg and the dose, expressed in terms of the salt, was that given at each individual injection. 5 rats were tested at each dose. For the experiments using the open field the same drug and dose injection schedule was used, with 6 rats being tested at each dose.

2.5. *Electroconvulsive shock (ECS)*

Electroconvulsive shock (30 mA, 50 Hz, 1 sec) was generated using a Grason Stadler 700 constant current shock generator and was delivered via ear-clip electrodes according to the same time schedule as the drug injections. Control rats had the electrodes placed in a similar manner but did not receive ECS. The effects of ECS on open field activity were investigated in a similar manner. With both methods 10 rats were tested per group.

2.6. *Deprivation of REM sleep*

Rats were selectively deprived of REM sleep according to the "island" method described by Mouret et al. (1969). After removal from the drying apparatus rats were placed individually in macrolon cages (42 × 27 × 18 cm) each containing a small circular platform (4.5 cm diameter) surrounded by water (10 cm deep). Food and water were available. They remained there under constant illumination during the 24 h between trials. A "stress" control group was treated similarly except that a larger platform (11.5 cm diameter) was used. Normal control rats were housed in their individual home cages during the 24 h inter-trial period.

Mouret et al. (1969) have reported that rats exposed to the larger platform (11.5 cm) showed a 50% reduction in the amount of slow wave sleep (SWS) without change in the proportion of REM, whereas those exposed to the smaller platform (4.5 cm) showed a similar reduction in SWS but a complete suppression of REM: the muscle atonia accompanying REM causes the rat to fall in the water thereby waking it up.

The effects of REM deprivation on open field activity were also investigated following a similar procedure. In both the swimming test and the open field test 6 rats were used per group.

2.7. *Enrichment of the environment*

After removal from the drying apparatus 6 "depressed" rats were exposed to an "enriched" environment by placing them in a dry water maze (Apelab) containing a variety of stimulus objects (paper rolls, a running wheel, blocks of wood) as well as 6 naive rats which had been placed in the enclosure just prior to the experiment. These rats were subsequently used as the experimental group in a parallel study of the effects of an "enriched" environment on open field activity. In addition to standard rat food pellets and water, rats in the enclosure had access to a varied diet containing fresh vegetable and meat matter. The rats were left in this environment during the 24 h between trials. Control rats were housed in their individual home cages.

A parallel experiment with the open field was performed comparing rats from the "enriched" environment with rats which were housed individually.

3. Results

3.1. *Behaviour without drugs*

Rats which were placed in the cylinders for the first time were initially highly active, vigorously swimming around, scrabbling at the walls or diving to the bottom apparently searching for an exit. After 2—3 min their activity began to subside being interspersed with phases of immobility (fig. 1) of increas-

TABLE 1

Effects of 5 sec and 15 min immersion in water 25°C) on open field activity. The rats (n = 10 per group) were tested immediately on being removed from the water. The number of quadrants entered during a 5 min period was counted. Differences from control were assessed statistically using the Dunnett test (2-tailed).

Treatment	Open field activity Mean (S.E.M.)
Control	18.5 (6.2)
Immersion (5 sec)	33.3 (5.8) [1]
Immersion (15 min)	4.1 (1.5) [1]

[1] $p < 0.05$.

ing length. After 5—6 min the duration of immobility reached a plateau where the rats remained immobile for approximately 80% of the time (fig. 2). When left in the cylinders for 15 min the rats were hypothermic on removal (mean: —3.15°C; S.E.: 0.16; n = 10) and showed reduced spontaneous motor activity (table 1).

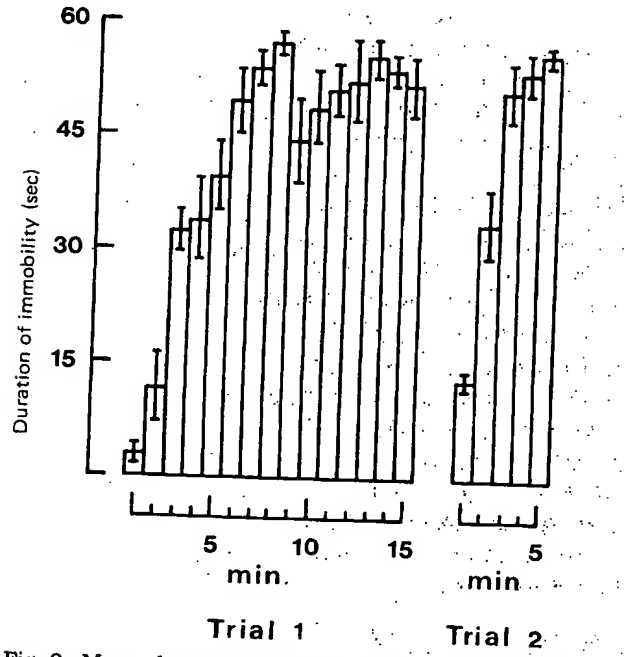

Fig. 2. Mean duration of immobility in sec per min ±S.E.M. (ordinate) as a function of time in the water (abscissa). On trial 1 rats (n = 10) were placed in the water for the first time. There was a 24 h interval between trials 1 and 2.

We have found that a single exposure of 15 min was sufficient to produce a relatively constant level of immobility in a subsequent test; on a second exposure to the cylinder, rats rapidly became immobile after a brief burst of activity and remained so for approximately 75% of a 5 min test (fig. 2). As can be seen from the control values in table 3, the immobility induced in this way was highly reproducible between different groups of rats on different days. We therefore adopted this procedure for the studies described below because it seemed to provide a suitable baseline for measuring the effects of treatments which decrease or even increase immobility.

3.2. Effects of different injection schedules of imipramine on immobility

Before beginning the drug studies described below, a series of experiments were under-

TABLE 2

Effects of different injection schedules of imipramine HCl on the total duration of immobility during a 5 min test. Differences from control were assessed statistically using the Dunnett test (2-tailed).

Imipramine injection schedule (h before test)	Dose (mg/kg i.p.)	Duration of immobility (sec) Mean (S.E.M.)
1 h	Control	250.8 (11.6)
	7.5	228.2 (26.9)
	15	158.8 (41.2)
	30	152.6 (20.0)
5 h	7.5	212.0 (18.3)
	15	168.8 (25.8)
	30	165.2 (29.9) [1]
24 h	7.5	251.4 (16.4)
	15	226.8 (5.6)
	30	192.4 (27.8)
24h + 1 h	7.5	180.4 (18.9) [1]
	15	180.4 (25.6) [1]
	30	88.6 (11.0) [2]
24 h + 5 h + 1 h	7.5	233.4 (17.8)
	15	124.0 (24.8) [2]
	30	78.6 (16.9) [2]

[1] $p < 0.05$.
[2] $p < 0.01$.

taken with imipramine to discover the optimal schedule of injections for producing a maximal pharmacological effect. Previous experience (Jalfre and Haefely, 1971) had indicated that multiple doses were superior to a single dose, as is also the case with the clinical use of antidepressant drugs.

Different groups of rats (n = 5) were given either 1, 2 or 3 injections of imipramine HCl (7.5, 15 or 30 mg/kg i.p.) as indicated in table 2. All animals received a total of three injections, 0.9% NaCl being given when a drug injection was not due.

The results (table 2) indicate that single i.p. injections of imipramine 1, 5 or 24 h before the test reduced immobility in a dose-dependent manner but that more pronounced and stable effects were observed when either 2 or 3 injections were given. The 3 injection schedule was chosen for subsequent experiments.

3.3. Effects of different drugs on immobility and on open field activity

The results obtained with different psychotropic drugs and are shown in table 3.

The tricyclic antidepressants tested (imipramine, desipramine, amitriptyline, nortriptyline) all caused a dose-dependent decrease in immobility; in the treated animals, in contrast to the controls, apparent attempts to escape persisted for a greater proportion of the 5 min test. These effects were observed despite a marked loss of muscle tonus at the highest doses and the fact that the same drugs under identical conditions of administration generally decreased open field activity.

The two monoamine oxidase inhibitors, iproniazid and nialamide, also reduced immobility in doses which otherwise diminished general motor activity. The disappearance of the antidepressant effect of iproniazid at 120 mg/kg seemed to be due to the pronounced hypotonia observed at this dose; the animals appeared quite limp and open field activity was greatly reduced.

Several different profiles of activity were observed with the compounds classed as atypical antidepressants. With fenfluramine, toloxatone and viloxazine, a clear and dose-dependent reduction in immobility was observed at doses which generally decreased open field activity. Biphasic effects occurred with iprindole and mianserin, a reduction in immobility with lower doses followed by a return to control levels and a loss of muscle tonus with higher doses. There was no correlation between the effects of these drugs on immobility and locomotor activity which was generally reduced. Qualitatively different effects were observed with nomifensine where the decrease in immobility observed at 10 mg/kg seemed to be due more to hyperactivity than to the persisting attempts to escape seen with the other antidepressants; open field activity was very variable with some animals showing amphetamine-like stereotyped head movements and little locomotion whereas in others locomotion was noticeably increased.

The two psychostimulants, d-amphetamine and caffeine, also reduced immobility but as with nomifensine, this effect appeared to be due to motor stimulation rather than to persistent attempts to escape; with d-amphetamine stereotyped head movements during swimming were observed at 1.5 and 3 mg/kg. Both drugs caused a marked increase in open field activity; stereotyped behaviour with d-amphetamine was, however, less apparent than in the swimming test.

Neither chlordiazepoxide nor diazepam affected the duration of immobility even in doses which produced noticeable ataxia. With chlordiazepoxide there was a tendency towards an increase in open field activity, an effect usually taken to reflect its anxiolytic properties. In contrast chlorpromazine and the reserpine-like compound Ro 4-1284 increased immobility and at the same doses decreased activity in the open field.

3.4. Effects of non-pharmacological treatments on immobility and on open field activity

The three non-pharmacological treatments investigated, electroconvulsive shock (ECS),

TABLE 3

Effects of different drugs on the total duration of immobility and on open field activity (number of quadrants entered) during 5 min tests. Differences from control were assessed statistically using the Dunnett test (2-tailed). [1] $p < 0.05$, [2] $p < 0.01$.

Drug	Dose (mg/kg i.p.)	Duration of immobility (sec) Mean (S.E.M.)	Open field activity Mean (S.E.M.)
Tricyclic antidepressants			
Imipramine HCl	control	250.8 (11.6)	73.3 (13.2)
	7.5	233.4 (17.8)	33.8 (11.4)
	15	124.0 (24.8) [2]	31.5 (10.7) [1]
	30	78.6 (16.9) [2]	27.5 (9.0) [1]
Desipramine HCl	control	214.2 (8.2)	67.2 (12.4)
	5	202.2 (14.3)	31.5 (14.3)
	10	174.8 (16.8)	23.0 (6.1) [1]
	20	98.8 (17.6) [2]	27.7 (12.9)
Amitriptyline HCl	control	245.4 (8.0)	63.2 (14.3)
	3.75	184.4 (5.5) [1]	48.0 (15.4)
	7.5	189.7 (18.9) [1]	27.3 (11.4)
	15.0	150.7 (22.2) [2]	38.8 (13.5)
Nortriptyline HCl	control	233.0 (19.4)	50.7 (13.0)
	5	202.2 (23.6)	15.8 (7.9) [1]
	10	185.6 (18.5)	23.3 (9.0)
	20	90.4 (18.4) [2]	9.0 (1.8) [2]
Monoamine oxidase inhibitors (MAOI)			
Iproniazid phosphate	control	249.2 (18.1)	56.2 (12.4)
	15	218.6 (7.0)	16.7 (4.4)
	30	196.8 (12.5)	27.7 (13.9)
	60	145.2 (15.8) [2]	28.3 (17.3)
	120	238.6 (22.7)	9.7 (4.7) [1]
Nialamide HCl	control	224.1 (9.6)	54.5 (17.0)
	40	231.2 (20.3)	19.3 (8.3)
	80	127.4 (29.6)	35.0 (19.8)
	100	83.4 (43.0) [2]	8.0 (5.1) [1]
Atypical antidepressants			
Fenfluramine HCl	control	248.2 (14.7)	57.2 (12.5)
	1.5	168.2 (18.6) [1]	73.5 (12.4)
	3	142.2 (12.5) [2]	32.2 (10.2)
	6	152.4 (32.8) [1]	20.7 (11.5)
	12	127.8 (17.0) [2]	11.0 (5.4) [1]
Iprindole HCl	control	243.1 (14.1)	53.8 (15.6)
	15	237.8 (15.9)	1.3 (0.8) [2]
	30	229.4 (21.3)	20.0 (6.1) [1]
	40	155.6 (24.8) [2]	13.3 (3.3) [1]
	60	260.6 (15.8)	6.5 (2.6) [1]
Mianserin HCl	control	210.6 (21.1)	43.8 (11.7)
	7.5	187.6 (11.2)	41.3 (17.6)
	15	140.0 (14.7) [2]	15.8 (5.7)
	30	147.2 (11.5) [1]	5.0 (2.1) [1]
	60	256.4 (16.6)	6.3 (3.8) [1]

TABLE 3 (continued)

Drug	Dose (mg/kg i.p.)	Duration of immobility (sec) Mean (S.E.M.)	Open field activity Mean (S.E.M.)
Nomifensine maleate	control	224.4 (15.6)	39.5 (12.0)
	2.5	162.4 (38.8)	52.0 (14.4)
	5	200.0 (38.6)	19.0 (4.9)
	10	38.6 (13.3) [2]	39.2 (17.9)
Toloxatone	control	227.0 (8.1)	75.8 (13.8)
	25	203.8 (6.7)	65.2 (6.6)
	50	168.0 (18.9) [2]	37.5 (16.0)
	100	128.4 (18.7) [2]	22.8 (8.1) [1]
	200	120.5 (12.3) [2]	21.3 (5.6) [1]
Viloxazine HCl	control	214.8 (12.1)	63.3 (14.2)
	12.5	205.6 (12.9)	30.8 (11.5)
	25	176.0 (23.2)	15.5 (6.9) [1]
	50	144.8 (15.3) [2]	34.7 (11.5)
Psychostimulants			
d-Amphetamine sulfate	control	202.8 (17.9)	49.8 (11.9)
	0.75	175.0 (9.2)	43.0 (5.5)
	1.5	85.4 (19.4) [2]	92.7 (10.7)
	3	25.4 (23.2) [2]	166.3 (16.7) [2]
Caffeine	control	219.6 (20.7)	50.5 (15.3)
	3.75	257.4 (8.7)	57.5 (15.2)
	7.5	177.2 (21.7)	65.2 (22.0)
	15	99.8 (21.0) [1]	97.8 (10.3) [1]
Minor tranquilisers			
Chlordiazepoxide HCl	control	237.6 (8.9)	65.8 (10.9)
	2	237.2 (16.0)	90.3 (9.5)
	4	225.6 (9.6)	85.2 (25.8)
	8	234.6 (9.4)	93.3 (18.1)
Diazepam	control	218.0 (10.7)	74.8 (15.9)
	0.5	224.6 (10.7)	56.3 (10.1)
	1	242.6 (12.7)	57.8 (15.0)
	2	216.6 (19.8)	46.3 (15.2)
	4	222.2 (21.1)	49.0 (15.5)
Major tranquilisers			
Chlorpromazine HCl	control	218.2 (2.2)	50.8 (8.4)
	0.75	220.6 (11.8)	11.3 (5.8) [1]
	1.5	224.0 (18.1)	10.2 (7.0) [1]
	3	263.0 (8.2) [1]	10.0 (9.2) [1]
Ro 4-1284	control	202.6 (7.3)	74.7 (20.2)
	1	271.2 (12.6) [2]	0.3 (0.1) [2]
	2	271.0 (12.0) [2]	0 (0) [2]
	4	257.8 (15.9) [2]	0 (0) [2]

[1] $p < 0.05$.
[2] $p < 0.01$.

TABLE 4

Effects of different non-pharmacological treatments on the total duration of immobility and an open field activity (number of quadrants entered) during 5 min tests. Differences from control were assessed statistically using the Dunnett test (2-tailed). [1] $p < 0.05$, [2] $p < 0.01$.

Treatment		Duration of immobility Mean (S.E.M.)	Open field activity Mean (S.E.M.)
Electroconvulsive shock (ECS)	control	238.7 (13.2)	26.8 (9.3)
	ECS	166.0 (24.7) [1]	21.8 (11.3)
Deprivation of REM sleep	control	229.9 (10.2)	75.2 (9.6)
	'stress'	231.1 (14.2)	112.3 (6.2) [1]
	REM depriv.	162.4 (11.6) [2]	133.8 (17.5) [1]
Enriched environement (EE)	control	232.0 (9.2)	99.1 (16.1)
	EE	182.7 (4.5) [2]	113.2 (11.0)

[1] $p < 0.05$.
[2] $p < 0.01$.

deprivation of REM sleep and "enrichment" of the environment all caused a significant reduction in immobility without greatly affecting open field activity (table 4).

With ECS, open field activity in the control group was lower than that usually observed (table 3) which may have been due to the multiple placement of the ear-clip electrodes (Materials and methods, section *2.5.*). There was, however, no difference between the open field activity of the ECS and of the control group suggesting that the decrease in immobility observed with ECS was relatively selective.

The results in the sleep deprivation studies also suggested fairly specific effects of REM deprivation on immobility. A decrease in immobility was observed in the REM deprivation group but not in the "stress" control group whereas both the "stress" control group and the REM deprivation group showed a significant increase in open field activity.

Finally, "enrichment" of the environment caused a significant decrease in immobility with only a slight and non-significant increase in open field activity.

4. Discussion

The present paper has described a new method for inducing in rats a behavioural state resembling depression by exposing them to a mildly aversive situation from which there is no possibility of escape. Preliminary experiments showed that prolonged exposure to such a situation produced increasing periods of virtually complete immobility which contrasted markedly with the vigorous attempts to escape observed when the animals were first introduced to the situation. These behavioural observations suggested that the animals, on finding that escape was impossible, gave up trying and resigned themselves to the experimental conditions. We hypothesised that the immobility observed reflected a state of lowered mood or hopelessness in the rat and predicted that immobility would be reduced by treatments which are known to be effective in alleviating depression in humans.

Our hypothesis received support from the results obtained in the experiments reported here not only with pharmacological agents but also with a variety of other treatments which are generally thought to be effective in depression. Furthermore several aspects of our findings suggest that the immobility observed in the water is a relatively specific depressive phenomenon. Firstly, it is apparent that the effects observed with the different

antidepressant treatments were not merely due to a stimulation of motor activity. Indeed, antidepressant effects as measured by reductions in immobility in the water generally occurred at doses which otherwise *decreased* activity as measured in the open field. Antidepressant effects could thus be clearly distinguished from the effects of psychostimulants which markedly increased open field activity. Even in the water the decrease in immobility observed with d-amphetamine and caffeine was accompanied by signs of a non escape-directed hyperactivity which, with the exception of nomifensine, was not seen with the antidepressants. Secondly it seems unlikely that the reductions in immobility observed were due to either diminished fear or drug-induced impairment of memory; chlordiazepoxide and diazepam which, in addition to their well known anxiolytic effects, are also thought to impair memory (Soubrié et al., 1976) were without effect whereas those agents which were effective in reducing immobility are not known for either their anxiolytic effects or their ability to impair memory in animal tests. Finally immobility was even enhanced by the two major tranquilisers chlorpromazine and the reserpine-like compound Ro 4-1284. While the increase in immobility may have been due to their pronounced sedative effects, it is possible that these drugs actually potentiated the depressive state induced in the experimental situation; it is widely accepted that reserpine-like compounds can cause clinical depressions in man (Schildkraut, 1972) and it has been suggested that depressive states can also result from treatment with neuroleptics (Helmchen and Hippius, 1967; De Alarcon and Carney, 1969).

The results obtained with the "atypical" antidepressants deserve special comment because these compounds, although clinically effective, differ in terms of both chemical structure and pharmacological activity from classical tricyclic antidepressants or monoamine oxidase inhibitors. Indeed the antidepressant activity of some of these "atypical" antidepressants would not be readily predictable on the basis of their pharmacological profile alone. For example iprindole, a tricyclic indole, although potentiating the stimulant effects of amphetamine, only weakly antagonises the hypothermia and ptosis induced by reserpine (Gluckman and Baum, 1969) and does not block the uptake of noradrenaline or serotonin (Ross et al., 1971; Fan et al., 1972; Koe, 1976). Nonetheless its clinical efficacy appears comparable with that of imipramine (Ayd, 1969; Rickels et al., 1973). A further example is the tetracyclic compound mianserin which possesses a profile of pharmacological and biochemical activity radically different from that expected of a classical tricyclic antidepressant. In animal tests the compound is strongly sedative having only slight and transient antireserpine activity (Van Riezen, 1972; Gouret et al., 1977) while antagonising the stimulant effects of amphetamine (Van Riezen, 1972). Although it inhibits the brain uptake of noradrenaline in vitro (Koe, 1976; Raiteri et al., 1976) mianserin increases the turnover of noradrenaline in vivo (Kafoe and Leonard, 1973) and is moreover a potent central serotonin receptor blocking agent (Jalfre et al., 1974; Van Riezen, 1972; Maj et al., 1976). Indeed the antidepressant potential of mianserin was not predicted from animal tests but was discovered virtually by chance during EEG studies in normal volunteers (Itil et al., 1972). Several double blind studies (Murphy, 1975; Wheatley, 1975; Coppen and Ghose, 1976; Vogel, 1976; Jaskari et al., 1977) have subsequently confirmed its antidepressant activity. A similarly incomplete antidepressant profile in pharmacological and biochemical tests is seen with the clinically active β-blocker derivative viloxazine (Vivalan Symposium, 1975; Floru et al., 1976; Moizeszowicz and Subira, 1977). Although it antagonises reserpine-induced hypothermia and sedation (Greenwood, 1975) viloxazine neither inhibits monoamine oxidase nor blocks the uptake of monoamines in brain (Mallion et al., 1972; Lippman and Pugsley, 1976; Koe, 1976). Another compound, nomifen-

sine, has also been reported to be an effective antidepressant (Angst et al., 1974; Acebal et al., 1976; Moizeszowicz and Subira, 1977) which strongly antagonises the effects of reserpine but, in contrast to tricyclic antidepressants, markedly stimulates motor activity (Hoffmann, 1973; Maj et al., 1976) and in addition to blocking noradrenaline uptake is a potent blocker of dopamine uptake in brain (Hunt et al., 1974; Samanin et al., 1975; Koe, 1976; Tuomisto, 1977). The relatively new compound toloxatone, an oxazolidinone derivative, also causes moderate antagonism of several effects of reserpine and potentiates amphetamine-induced stereotypies at doses which are otherwise markedly sedative (Gouret et al., 1973; Coston et al., 1975; Gouret and Raynaud, 1975). In contrast to the above-mentioned compounds toloxatone appears to act through a relatively specific inhibition of type A monoamine oxidase but unlike other MAOIs in clinical use this inhibitory effect is reversible and of short duration (Kan et al., 1977). Preliminary studies indicate that toloxatone is clinically effective (Martin, 1973; Suttel and Duplan, 1973). The remaining compound in the series of "atypical" antidepressants, fenfluramine, a non-stimulant derivative of amphetamine (LeDouarec and Neveu, 1970), is best known for its anorexic properties (Pinder et al., 1975). Fenfluramine nonetheless possesses other pharmacological properties, e.g. the release of serotonin from central storage sites (Trulson and Jacobs, 1976) and the inhibition of its uptake into blood platelets (Wielosz et al., 1976). These properties are not inconsistent with antidepressant activity and indeed such activity has recently been reported in man (Murphy et al., 1976). The fact that all these clinically effective compounds showed antidepressant effects in our test procedure despite their widely differing modes of action provides additional support for our hypothesis that the immobility observed reflects a state of depression in the rat. It is noteworthy that the present procedure, which is based on behavioural rather than biochemical concepts, is the first animal test model which clearly predicts an antidepressant action for mianserin.

Further evidence for the validity of our model is provided by the positive findings obtained with non-pharmacological antidepressant treatments. Electroconvulsive treatment, despite increasing caution in its use, is still widely accepted as being the most effective and rapid of existing treatments for endogenous depression (Ilaria and Prange, 1975). Similarly, sleep deprivation, although less effective in the long term, is widely used for the treatment of various kinds of depression (Post et al., 1976; Rudolf et al., 1977) especially as an adjunct to more classical pharmacotherapy (Loosen et al., 1976). Furthermore there is increasing evidence that the therapeutic effects of sleep deprivation are specifically related to deprivation of REM sleep (Vogel, 1975; Schilgen et al., 1976). The present results provide added evidence for this in that they show a highly selective effect of REM sleep deprivation in reducing immobility in the rat. The fact that exposure to an "enriched environment" also selectively reduced immobility in the rat, although intuitively predictable, is more difficult to interpret in view of the absence of systematic research in this area. Nonetheless the positive findings obtained heuristically suggest that further research into the role of environmental and social factors in antidepressant therapy would be worthwhile (Benson, 1975).

We conclude from the findings described and discussed above that our test procedure reproduces some aspects of human depression in the rat. It is therefore of interest to seek parallels between our model and other behavioural models described in the experimental literature. The most striking of these is the depressive syndrome occurring in young rhesus monkeys after separation from their mothers, their age mates or after confinement in "vertical chambers" (Harlow and Suomi, 1974). The syndrome consists typically of two phases, first a high level of agitation and vocalisation (protest) followed by a marked

reduction in general activity, together with increases in huddling and self-clasping (despair). A similar behavioural pattern has been observed in young dogs (Scott et al., 1973) and resembles the anaclitic depression described in young children who are separated from their parents (Bowlby, 1977). Although the time scale is greatly reduced, it is tempting to see a resemblance between these protest and despair reactions and the behaviour observed in our test procedure where a short period of frantic activity is followed by periods of immobility of increasing duration. Another model which is currently gaining wide interest is Seligman's "learned helplessness" (Seligman, 1975). According to this model animals and even humans, when exposed to aversive situations over which they have no control, are subsequently less able to respond adaptively to traumatic events. For example, dogs when given inescapable electric shocks were unable on a later occasion to learn the responses necessary to escape from shocks of similar intensity (Seligman and Maier, 1967). Similar deficits have been observed in experiments with man and have been accompanied by mood changes similar to those occurring in clinical depression (Miller and Seligman, 1975). Seligman calls the behavioural disturbance "helplessness" and suggests that "helplessness" occurs because the organism has learned that attempting to escape is futile. Although the present experiments do not test whether rats inescapable exposed to water are subsequently less able to learn to escape, there is an obvious parallel between the kind of situation investigated in our procedure and that described by Seligman. If so, Seligman's findings suggest that our experimental situation may well induce feelings of helplessness in the rat whereas our findings provide pharmacological evidence that "learned helplessness" is indeed related to depression.

In conclusion, we think that our test procedure represents a new approach to the study of depression in animals and is both pharmacologically and behaviourally valid. Because of its simplicity it should lend itself readily to experimental manipulation; studies of the biochemical and electrophysiological correlates of immobility are already in progress in our laboratory. Furthermore the fact that several atypical antidepressant compounds, for example mianserin and iprindole, show a clear antidepressant activity in the test procedure, raises the intriguing possibility that the present method may be capable of discovering new types of antidepressant agents hitherto undetectable using classical screening tests.

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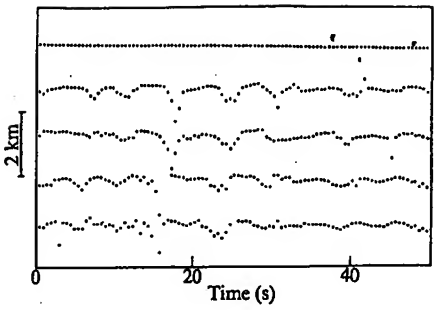

Fig. 2 Apparent variations of group heights recorded at four aerials and the corresponding phase height variations (top trace).

considered and especially when the data show unequal changes in group and phase path. Oscillations in the group path show changes of the order of ±200 m while inspection of phase path records show the oscillations to be of the order of ±10 m. Other effects such as Doppler shift due to atmospheric winds can also affect the apparent horizontal velocity. Consequently, it is difficult to confidentially deduce trace velocities from the data.

Second, Munro and Whitehead consider that if the oscillations represent real changes in group height, then corresponding changes in phase height would occur and that phase height experiments would have previously detected such waves. However this argument ignores the possibility of retardation effects which can be important. Calculations of the effects of irregularities on group and phase path show that changes in phase path can typically be about a tenth of the change in group path[3,4].

Finally, if the effect is produced by interfering echoes, there is an unusual effect in that the oscillations are restricted to a relatively limited range of frequencies. Although this does not preclude interference as an explanation of these oscillations, it suggests that the interference mechanism will not be simple in nature. If the irregularities producing interference were moving vertically at the speed assumed by Munro and Whitehead, their progression through the layer should have been observed in Fig. 1. If the irregularities move horizontally, velocities of up to 300 m s^{-1} are implied which again means that sound or gravity waves might be involved.

It should be noted that the definition of the quantity θ used by Munro and Whitehead is strictly only valid for a mirror reflector and the more general definition is $\theta = 4\pi\Delta h_p f/c$.

Thus it seems that although interference may be the explanation there are problems with the simple interference approach, but more importantly, invoking interference does not preclude the problem of identifying the type of irregularities responsible.

Clearly more work needs to be done to satisfactorily explain the oscillations and further studies are being carried out at La Trobe University.

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Swimming rats and human depression

WE believe that the rat swimming test of Porsolt *et al.*[1] is a valuable contribution to drug screening methodology and we are now using it in our present work. Sprague-Dawley rats are individually tested in a narrow plexiglass cylinder (height 40 cm, diameter 18 cm) containing 15 cm of water. Initially, the rats swim vigorously but later make only the movements necessary to keep their heads above water. In drug screening, naïve animals are given a 15-min swim on the first day, then dried and given the first of three spaced drug injections. They undergo a 5-min swimming test 24 h later in which the period of relative immobility is timed. As clinically effective antidepressant drugs are selectively identified in this way, Porsolt *et al.* conclude that the behaviour evaluated represents 'lowered mood' and 'despair' in the animals. We believe this interpretation is contrary to other behavioural activity seen in the test which they do not report.

We replicated their control group condition for the equipment described, strain used (167–230 g), most common subgroup size ($n = 5$), water temperature and procedure. We also made further observations (adjusted for time) comparing day 1 and day 2 tests. We confirm their finding with regard to the behavioural change seen but wish to describe it more fully. The rats quickly learn to touch bottom with their tails and hind feet. They are then able to maintain a position in which they prop themselves against the side of the cylinder without the energy expenditure required in swimming. This we consider to be an adaptive response.

We make the following points: (1) implicit in the two-day test is the idea that behaviour may change in the absence of any other independent variable. Early in the day 1 test, two indicators of behavioural disturbance were seen: diving[2–4] and headshaking. Four of our subjects dived on the first day, none on the second. All showed headshaking on day 1, only one did so on day 2. Again, we interpret these changes as being adaptive in this situation. (2) If the immobility response represents 'despair', it should be maintained once adopted, that is, if the animal has given up, it will not try again. Instead, we found that all animals switched back to swimming and back again to immobility on both days. The count for such changes in the 5-min interval was: day 1, $\bar{\chi} = 3.6$; day 2, $\bar{\chi} = 3.8$; $t_{dep} = 0.25$. (3) Finally, emotional defaecation has been used as a measure of fearfulness in a variety of experimental contexts[5–7]. Bolus counts for our subjects gave these results: day 1, $\bar{\chi} = 5.6$; day 2, $\bar{\chi} = 3.0$; $t_{dep} = 2.23$, d.f. $= 4$, $P < 0.05$ (one-tailed test). This supports the idea that having been rescued on day 1, the rats were less fearful on day 2.

We conclude that the animals are making adaptive responses to a stressful situation. Drug effects which reduce immobility and increase swimming time may predict which new antidepressant drugs will be effective. Such a result will be more convincing when concomitant behaviour is also observed and reported. Even then, such a result should be interpreted cautiously. The swimming test does not provide a model resembling depressive illness in human beings.

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PORSOLT AND JALFRE REPLY—The description of the rat's behaviour provided by Hawkins *et al.* corresponds well with what we have observed ourselves[1,2];

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the rats on first exposure to the apparatus show highly active swimming, headshaking, diving and defaecating frequently. With prolonged exposure, and particularly during the 5-min test on the second day, the vigorous activity declines, being interspersed with phases of immobility of increasing duration.

We do not, however, agree with their conclusion that the rats are merely making an adaptive response to a stressful situation which is unrelated to a state of lowered mood. Although it is evident that the rats do adapt to the situation in that their initial agitated behaviour rapidly subsides, we see no reason to exclude *a priori* the possibility that they may at the same time also be feeling depressed. Three lines of evidence would support this latter proposition. First, it is known that exposure to inescapable traumatic situations can induce symptoms of depression in man and higher animals[3,4]. Second, our results show that a variety of clinically effective antidepressant drugs selectively decrease immobility whereas other drug classes do not[1,2]. Finally, we have shown that immobility is also reduced by nonpharmacological treatments such as electroconvulsive shock and deprivation of REM sleep[2], procedures which are generally accepted to be effective in the treatment of human depression[5,6]. A further point with which we disagree is the assumption of Hawkins *et al.* that for immobility to represent despair, it should be maintained once adopted. It seems to us more reasonable to expect the animal to oscillate between attempts to escape and immobility, with immobility predominating as the inescapability of the situation becomes more apparent, which is what both we and Hawkins *et al.* observe.

In general, we do not claim to have produced a model of human clinical depression in the rat. Nonetheless, our results encourage us to believe that our procedure provides an animal model of at least some aspects of depressed mood which is readily amenable to experimental manipulation.

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Effect of osmolarity on quantal size

VAN DER KLOOT reports[1] that he was unable to detect an amplitude change in miniature end plate potentials as a result of the imposition of osmotic changes in the incubation medium of the nerve–muscle preparation. He argues that his failure excludes a release mechanism depending on the free acetylcholine (ACh) in the cytoplasm because the change in osmolarity should have changed the terminal volume which should then have changed the free ACh concentration. This would have been then detectable as a change in the amplitude of the miniature end plate potential.

Unfortunately, he did not measure the change in the terminal volume so we have no idea how successful his procedure was in overcoming the various compensatory mechanisms for maintaining cell volume.

More critically, he neglected to measure the free ACh concentration. The factors which control this are unclear but it is certain that the concentration of free transmitter can change significantly in response to functional demands within five seconds[2]. The activity of choline acetyltransferase in neuromuscular junction terminals is sufficient in appropriate conditions to synthesise (or degrade; it is fully reversible) the whole transmitter store within the 20-s period it took him to change the osmolarity and repenetrate the muscle[3].

I am afraid we are no further forward unless Van der Kloot can supply direct evidence that the free ACh concentration changed in the way he thinks it did.

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VAN DER KLOOT REPLIES—The changes in the volume of frog motor nerve terminals in hypertonic solutions were described by Clark[1]. The nerve terminals surely shrink appreciably in hypertonic solutions.

The experimental demonstration of a swift mechanism for the homeostasis of free acetylcholine in nerve terminals, operating over massive changes in concentration, like that proposed by Marchbanks, would be of substantial interest in itself and certainly, as I pointed out in my report, could account for the observations within the framework of the gated channel hypothesis for quantal acetylcholine release. The major problem that would remain is how the diffusion of charged acetylcholine cations through membrane channels could be independent of the potential across the membrane.

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Use of ADTN to define specific ^{3}H-spiperone binding to receptors in brain

THE report of Leysen *et al.*[1] concerning the binding of the relatively new radiolabel ^{3}H-spiperone to both dopamine (DA) and 5-hydroxytryptamine (5-HT) receptor sites suggests that caution is needed in using this radioligand for DA receptor binding

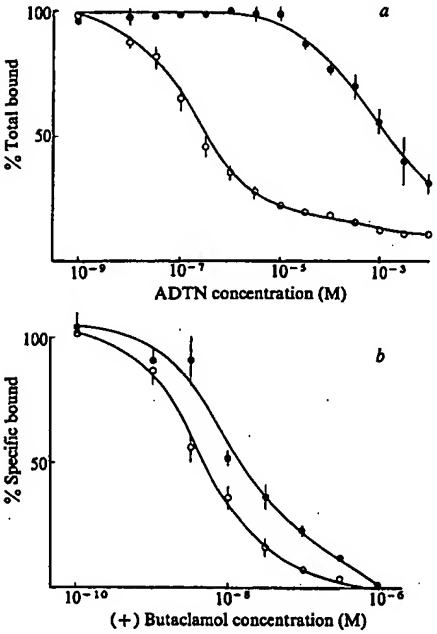

Fig. 1 Effect of ADTN (*a*) and (+)-butaclamol (*b*) on the binding of ^{3}H-spiperone in rat brain striatal (○) and medial frontal cortex (MFC) (●) homogenates. Tubes contained 0.50 nM and 0.25 nM ^{3}H-spiperone for the medial frontal cortex (MFC) and striatum, respectively, these represented the K_d values of saturable binding sites in the two brain regions. Each point represents the mean ± s.e.m. of 4–10 determinations from 2–5 separate experiments. In *a*, the results are expressed as % of total ^{3}H-spiperone binding, corrected for filter blanks. In *b*, results are expressed as % of 'specific' ^{3}H-spiperone binding, defined as that displaced by 1 μM (+)-butaclamol.

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Research report

Prenatal exposure to diazepam and alprazolam, but not to zolpidem, affects behavioural stress reactivity in handling-naïve and handling-habituated adult male rat progeny

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Abstract

A gentle long-lasting handling produces persistent neurochemical and behavioural changes and attenuates the impairment in the behavioural reactivity to novelty induced by the prenatal exposure to diazepam (DZ) in adult male rat progeny. This study investigated the consequences of a late prenatal treatment with three GABA/BDZ R agonists (DZ) alprazolam (ALP) and zolpidem (ZOLP)), on different stress-related behavioural patterns, in non-handled (NH), short-lasting handled (SLH) and long-lasting handled (LLH) adult male rats exposed to forced swim test (FST), acoustic startle reflex (ASR) and Vogel test (VT). The effects on motor activity were evaluated in the open field and in the Skinner box. The seizure sensitivity to picrotoxin (PTX) was investigated as an index of the functional state of GABA/BDZ Rs. A single daily s.c. injection of DZ (1.25–2.50 mg/kg) and ALP (0.125–0.250 mg/kg) over gestational days 14–20 induced a decrease in immobility time in the FST in NH rats, no change in SLH rats and an increase in LLH rats; DZ induced an increase in the peak amplitude of the ASR in NH rats, no change in SLH rats and a reduction in LLH rats; ALP was ineffective in all groups. DZ and ALP reduced the number of punished licks in the VT in NH, SLH and LLH rats while the unpunished licks were not modified. DZ decreased locomotion and the lever pressing responses while ALP increased them. DZ and ALP increased the seizure sensitivity to PTX (2.5–4.0 mg/kg i.p.). These findings indicate a convergence on anxiety-related behaviours in the effects of prenatal exposure to DZ and ALP and a differentiation on motor activity. Long-lasting handling was able to overcompensate the increased behavioural stress reactivity induced by the prenatal exposure to DZ and ALP.

Theme: Neurotransmitters, modulators, transporters, and receptors

Topic: Behavioral pharmacology

Keywords: Rats; Prenatal treatment; BDZ R agonist; Handling; Stress-related behavior

1. Introduction

The diversity of the behavioural effects of compounds acting on the GABA/benzodiazepine receptors (BDZ Rs) reflects the ubiquitous expression of these receptors in the brain and the intrinsic selectivity and efficacy of ligands which bind on different GABA/BDZ R subtypes [29,30,44]. Moreover, due to the different subunits expressed by the GABA/BDZ Rs in the central nervous system, some ligands act as partial agonists on certain receptor subtypes and as full agonists on others [40,53,66]. Examples of GABA/BDZ R agonists with different receptor selectivity and distinct behavioural profiles are diazepam (DZ), alprazolam (ALP) and zolpidem (ZOLP). DZ is a benzodiazepine with low affinity for GABA/BDZ Rs [4,28,47], which induces anxiolytic-sedative and myorelaxant effects at partially overlapping dose ranges [5]. Alprazolam (ALP) is a triazolobenzodiazepine with

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high affinity for specific GABA/BDZ R subtypes [4,27]; it produces anxiolytic, antipanic, antidepressant and weak sedative effects [16,20,50,58,59]. ZOLP is an imidazopyridine, unrelated to benzodiazepines, which shows a high selectivity and affinity for the BDZ R subtype ω1 [2,3,8,28,41], strong sedative, and weak anxiolytic effects [19,42,51,52,66].

In rats, prenatal exposure to benzodiazepines during the ontogenic development of the brain results in a decrease in maturation and in a down-regulation of GABA/BDZ Rs [10,26,38,43,46]. These changes are associated with altered behavioural reactivity, modified electrographic brain response, impaired capability of the animals to adapt to environmental challenges and deficit in spatial exploration [13–15,37–39,57].

The complexity of the effects following prenatal benzodiazepine exposure on anxiety-related behaviours cannot be explained by a single factor, since prenatal exposure to DZ has been found to determine both anxiogenic and anxiolytic effects [14,15,23,36,42,44,57].

Several aspects of the functioning of the GABA system can be affected by environmental stimuli. In rats, a repeated gentle handling results in an increased response of GABA/BDZ Rs in the brain [9,18]. When exposed to behavioural testing, handled rats are less anxious and exhibit an attenuated reactivity compared to non-handled animals [1,11,34]. Moreover, handling during infancy produces long-lasting neurochemical changes on adult rats and attenuates some behavioural deficits which emerge with age in handled-naive rats [48,49]. One could then hypothesize an interaction between the effects of environmental stimuli, such as handling, and the action of drugs acting on the GABA/BDZ R complex. Indeed, an early long-lasting handling is able to partially counteract the changes induced by prenatal exposure to DZ on the behavioural reactivity to novelty in adult rats [15].

The purpose of this study was to investigate the consequences of late prenatal exposure to DZ, ALP and ZOLP on different stress-related behavioural patterns in the male rat, and whether handling could correct the drug-induced alterations.

The effects of late prenatal exposure to the three different GABA/BDZ R agonists were examined in non-handled, short-lasting handled and long-lasting handled adult male rat offspring. Three behavioural tests, based on different stressors, were employed: forced swim test (FST), acoustic startle reflex test (ASR) and Vogel test (VT).

Because prenatal administration of DZ, ALP and ZOLP might induce motor dysfunction, interfering with the effects exerted on behavioural stress reactivity, a secondary aim of this research was to investigate the influence of prenatal exposure to DZ, ALP and ZOLP on locomotor activity in open field, as an expression of a non-specific behavioural pattern. Furthermore, lever pressing responses in a reward-facilitated operant schedule of water reinforcement were also assessed, as an expression of a specific motivated behaviour. Finally, the seizure sensitivity to picrotoxin (PTX), a specific GABA/BDZ R antagonist [33], was evaluated as an index of the functional state of GABA/BDZ Rs [12].

2. Methods

2.1. Animals

Male Wistar rats, weighing 280–300 g, were used. Animals were housed at constant temperature (20±2 °C) and relative humidity (55±10%) on a regular light–dark schedule (light 08:00–20:00 h). Food and water were available ad libitum.

2.2. Pharmacological treatment

Pairs of primiparous females (age ~120 days) were mated with a single male (age ~150 days). The day on which sperm was detected in the vaginal smear was designated gestation day 1. Pregnancy was determined by weighing and palpation. The pregnant dam's weight on gestational day 14 was ~300 g. From gestational day 14 through gestational day 20, nine groups of mothers received a single daily s.c. injection of DZ (1.25 or 2.50 mg/kg), ALP (0.125 or 0.250 mg/kg), ZOLP (4.0 or 8.0 mg/kg), or respective vehicle solutions. The doses of DZ, ALP and ZOLP administered to the pregnant rats were comparable to doses generally prescribed for pregnant women.

At birth all litters were culled to 10 pups: five females and five males. DZ-, ALP- and ZOLP-exposed pups were not fostered at birth by untreated dams, since it has been shown that the consequences of prenatal exposure to benzodiazepines are not influenced by fostering [10]. Pups, randomly selected from 3–4 litters, were weighed once weekly and weaned at postnatal day 28. At this time males were housed in groups of six per cage and, at postnatal day 60, in groups of three per cage. No differences in mortality or weight gain were observed between prenatal DZ/ALP/ZOLP- and vehicle-exposed rats.

2.3. Handling procedure

At post-natal day 28, a first group of rats was submitted to daily handling, Monday through Saturday, until the third month of age. The offspring were picked up and gently handled for 3 min before returning to their home cage (long-lasting handled (LLH) group). The offspring of a second group were submitted to handling 5 days before testing (short-lasting handled (SLH) group). The offspring of a third group were left undisturbed until testing at 3 months of age (non-handled (NH) group). Handling ses-

sions were always performed in the same room by the same experimenter.

2.4. Behavioural tests

On the test day, animals were brought into the laboratory and allowed to acclimatise for at least 30 min before each session. The effects of the prenatal exposure to two doses of DZ, ALP and ZOLP were evaluated on FST, ASR and VT. The highest doses of DZ, ALP and ZOLP were used to study motor activity in the open field and in the Skinner-box and seizure sensitivity to PTX since the lower doses did not reach significance. Rats from each of the three prenatally vehicle-treated groups were processed in a single control group (see Results). Different groups of animals were used for each test employed. SLH rats were employed in the open field and in operant behaviour tests.

All the experiments were performed in a sound-isolated chamber, between 10:00 and 14:00 h, in February and March, by an experimenter unaware of the prenatal treatment.

2.5. Forced swim test

NH, SLH and LLH rats ($N=12$) were placed in a plexiglass cylinder (40 cm high, 18 cm inside diameter) containing 5–6 l of clean water, depending on the rat size, maintained at 22±1 °C. Immobility time was recorded, for 10 min, by a trained observer. Rats were judged to be immobile when they remained floating into the water, just keeping their head above the water.

2.6. Acoustic startle reflex test

The ASR was measured with a Responder-X apparatus (Columbus Instruments, USA) employing NH, SLH and LLH rats ($N=12$). The peak amplitude of the response was recorded and displayed on a PC. The startle device was placed into a ventilated, sound-attenuated, dark chamber. On the test day, each rat was placed in a stainless-steel grid floor device (28×16×15 cm). The startle stimulus consisted of a 110 dB, 8 kHz tone superimposed on a continuous 50 dB white noise background; the stimulus duration was 200 ms, with a fixed 10-s interval. Sound levels in the test chamber were measured with a Bruel & Kjaer 2209 sound level meter. The maximum force exerted by the animal on the grid floor during the 200 ms period was designated as peak amplitude. Amplitude was measured in units; over the range of 60 to 550 g (one unit=2.1 g of force); the maximum output was 255 units. Each session consisted of 11 trials. According to Rigdon and Weatherspoon [54] the first trial was discarded.

2.7. Vogel test

NH, SLH and LLH rats ($N=12$), deprived of water for 24 h, were allowed to explore and drink freely for 15 min to become familiar with the apparatus (45×25×25 cm) (Anxiometer, Columbus, USA). The cage contained a drinking tube connected to an external 50-ml burette filled with tap water. The animals were then returned to their home cage and deprived of water for an additional 24 h. The following day the rats were again placed in the test cage. The trial period began when the rat's tongue came in contact with the drinking tube for the first time. Rats were allowed 1 min of unpunished licking. The following 3 min at the 20th lick they received 0.25 mA electric shock of 0.3 s duration, delivered to the tongue. The number of punished and unpunished licks was recorded during 4-min sessions.

2.8. Open field test

Locomotor activity was measured as total distance travelled (TDT) in a dimly illuminated chamber with a contrast-sensitive video-tracking system in SLH rats ($N=16$) [64]. The apparatus used a square box (44 wide×44 long×19 cm high), whose adjacent perpendicular sides had 15 infrared emitters separated by 2.65 cm. TDT was recorded and displayed on a PC. Each session lasted 10 min, beginning 1 min after rat placement in the box. The test was performed across five sessions, with an interval of 5 days between the sessions.

2.9. Operant behaviour test

The lever pressing responses were studied in SLH rats ($N=6$). The animals were water-deprived for 2 days, placed in a Skinner-box cage (Ralph Gerbrand Model) and trained to press a lever in order to obtain water. After a shaping-period with rewarded responses, rats were trained progressively to respond according to a variable ratio 30% (RV 30) schedule of reinforcement. Five sessions of 30 min were recorded when RV 30 bar-pressing rate was achieved.

2.10. Sensitivity to picrotoxin

Prenatally-exposed (vehicle, DZ, ALP and ZOLP) SLH rats ($N=10$) were examined to test their sensitivity to PTX (1.0, 2.5, 4.0 mg/kg i.p.). Following PTX treatment, rats were placed in separate cages and observed for 30 min. The incidence of generalised seizures was recorded with the different doses of PTX.

2.11. Drugs

DZ was given as Valium (Roche, Milan, Italy)—40% v/v propylene glycol, 10% v/v ethyl alcohol in water; ALP (Upjohn, Kalamazoo, USA)—suspended in 1% aqueous solution of carboxymethylcellulose; PTX (Sigma-Aldrich, Italy) and ZOLP (Synthelabo, Limito, Italy) were

dissolved in a 0.9% NaCl solution. All drugs were administered in a constant volume of 1 ml/kg of body weight.

2.12. Statistics

Statistical evaluation of FST, ASR and VT were performed with two-way ANOVA followed by a post hoc Tukey's test. Concerning the open field test (OFT) and the operant behaviour test, a single Student's *t*-test has been performed for each experimental session.

3. Results

One-way ANOVA on data concerning the effects of the prenatal exposure to the three vehicles in FST, ASR, VT, OFT and operant behaviour test showed no significant differences for each group of rats

FST
NH, SLH, LLH: *P* level = 0.75–0.82–078
ASR
NH, SLH, LLH: *P* level = 0.94–0.91–0.69
VT
NH, SLH, LLH unpunished licks: *P* level = 0.78–0.80–0.90
NH, SLH, LLH punished licks: *P* level = 0.94–0.93–0.97
OFT: *P* level = 0.95
OPERANT BEHAVIOUR TEST: *P* level = 0.95.

3.1. Forced swim test

The results of two-way ANOVA performed on handling and drug exposure as independent variables are shown in Table 1A. Except for prenatal ALP exposure and for handling and prenatal ZOLP exposure, the results indicate that handling and prenatal exposure to different compounds or the interaction between these factors are significant.

Table 2
Effects of prenatal exposure to vehicle, DZ, ALP and ZOLP on the immobility time in the FST in adult rat offspring

Prenatal treatment (mg/kg)	NH	SLH	LLH
Vehicle	152±5	161±6	182±6°
Diazepam			
1.25	145±4	166±4°	199±7*°
2.50	140±4*	169±5°	209±8*°
Alprazolam			
0.125	148±5	162±5°	188±6°
0.250	142±4*	166±5°	192±5*°
Zolpidem			
4.0	155±7	162±6	187±6°
8.0	150±5	167±4°	189±6°

Each value represents the mean±S.E.M. from 12 rats. *$P<0.05$ vs. respective controls; °$P<0.05$ vs. respective NH group.

The effects of prenatal treatment with DZ, ALP and ZOLP on immobility time in the FST are shown in Table 2. Short-lasting handling and long-lasting handling induced a significant increase in immobility time in vehicle-, DZ-, ALP- and ZOLP-exposed rats, compared to non-handling. Compared to vehicle-exposed rats, the highest dose of DZ and ALP induced a significant decrease in immobility time in NH rats, while prenatal DZ and ALP did not induce significant changes in SLH rats; conversely, they significantly increased immobility time in LLH rats. Prenatal

Table 1
Results of the two-way ANOVA performed on handling (1) and drug-exposure (2) (DZ, ALP and ZOLP) as independent variables[a]

Effects	DZ			ALP			ZOLP		
	df	*F*	*P*-level	df	*F*	*P*-level	df	*F*	*P*-level
A									
1	2	664.81	0.000001*	2	513.31	0.000001*	2	289.16	0.000001*
2	2	15.30	0.000002*	2	1.67	0.194145	2	4.64	0.011809*
1:2	4	30.66	0.000000*	4	9.99	0.000001*	4	2.12	0.084055
Residuals	99			99			99		
Total	107			107			107		
B									
1	2	289.14	0.000001*	2	95.41	0.000001*	2	33.92	0.000001*
2	2	0.53	0.592587	2	0.22	0.804022	2	4.95	0.008906*
1:2	4	34.55	0.000001*	4	6.50	0.000110*	4	0.62	0.648530
Residuals	99			99			99		
Total	107			107			107		
C									
1	2	117.11	0.000001*	2	114.44	0.000001*	2	81.29	0.000001*
2	2	157.70	0.000001*	2	42.35	0.000001*	2	3.84	0.024681*
1:2	4	3.39	0.012103*	4	1.11	0.357073	4	0.98	0.421337
Residuals	99			99			99		
Total	107			107			107		

[a] The effects marked with an asterisk are significant with $\alpha = 0.05$. A, FST; B, ASR; C, Vogel test.

exposure to ZOLP was ineffective in all experimental conditions.

The effects of handling on the performance in the FST of DZ-, ALP- and ZOLP-treated rats are shown in Fig. 1. The inverted inclination of the curves in NH and LLH rats indicates that long-lasting handling is able to overcompensate the effects of prenatal DZ on the FST.

3.2. Acoustic startle reflex test

The results of two-way ANOVA performed on handling and drug exposure as independent variables are shown in Table 1B. The results indicate that handling and prenatal exposure to the different compounds and the interactions between these factors are significant, except for prenatal DZ/ALP exposure and the handling–prenatal exposure interaction. The effects of prenatal treatment with DZ, ALP and ZOLP on the peak amplitude of the ASR are shown in Table 3. Compared to non-handling, long-lasting handling induced a significant reduction in the peak amplitude of ASR in vehicle, DZ-, ALP- and ZOLP-exposed rats. Compared to respective controls, NH DZ-exposed rats showed a significant increase in the peak amplitude of ASR, while no significant changes were observed in NH ALP-exposed rats. Prenatal DZ and ALP did not produce significant effects on SLH rats. LLH DZ-exposed rats displayed a significant reduction in the peak amplitude of ASR, while ALP did not induce significant variations. No significant differences were observed between vehicle- and ZOLP-exposed NH, SLH and LLH rats. The effects of handling procedure on the peak amplitude of ASR of DZ-, ALP- and ZOLP-treated rats are shown in Fig. 2. The inverted inclination of the curves in NH and LLH rats indicates that handling is able to overcompensate the effects of prenatal DZ on the ASR.

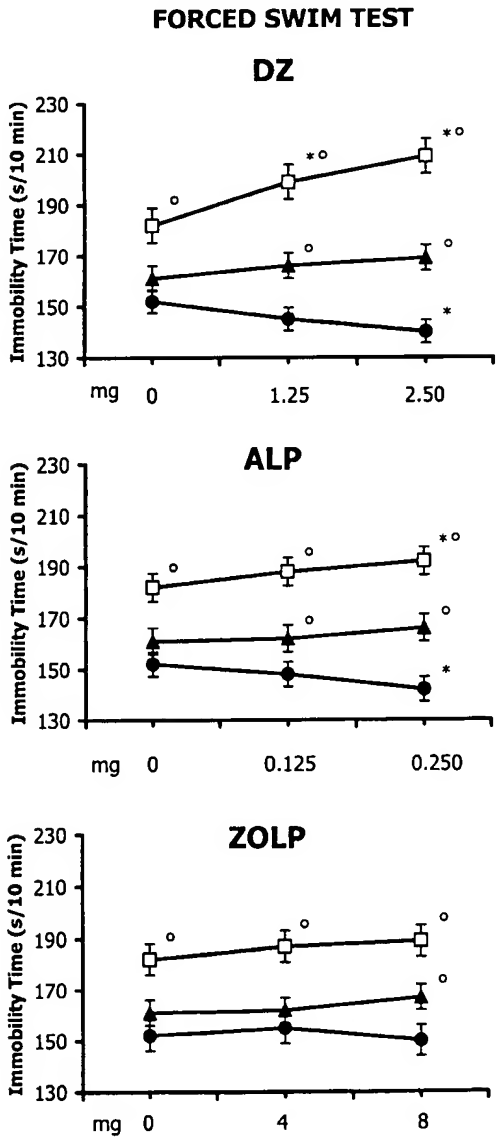

Fig. 1. Effects of prenatal exposure to vehicle, DZ, ALP and ZOLP on the FST. Each value represents the mean±S.E.M. from 12 rats. *$P<0.05$ vs. respective controls; °$P<0.05$ vs. respective NH group. NH ●, SLH ▲, LLH □. NH, non-handled; SLH, short-lasting handled; LLH, long-lasting handled.

3.3. Vogel test

The data of two-way ANOVA performed on handling and drug exposure as independent variables are shown in Table 1C. The results indicate that handling and prenatal drug-exposure or the interaction between these factors are significant, except for the handling– ZOLP exposure and handling–ALP exposure interactions.

The effects of prenatal exposure to DZ, ALP and ZOLP on unpunished and punished licks are shown in Table 4. Compared to non-handling, long-lasting handling induced a significant increase in the number of punished licks in control rats. Compared to controls, rats exposed to DZ showed a significant reduction in the number of punished licks; this effect was more evident in NH and SLH rats. Similarly, exposure to the highest dose of ALP induced a decrease in licking in NH, SLH and LLH rats, although the

Table 3
Effects of prenatal exposure to vehicle, DZ, ALP and ZOLP on the peak amplitude of the ASR in adult rat offspring

Prenatal treatment (mg/kg)	NH	SLH	LLH
Vehicle	64±4	58±5	53±4°
Diazepam			
1.25	73±6*	61±6°	40±5*°
2.50	79±4*	64±6°	35±5*°
Alprazolam			
0.125	68±6	62±6	47±8°
0.250	70±5	64±7	44±6°
Zolpidem			
4.0	62±6	55±7	51±6°
8.0	61±7	55±7	46±6°

Each value represents the mean±S.E.M. from 12 rats. *$P<0.05$ vs. respective controls; °$P<0.05$ vs. respective NH group.

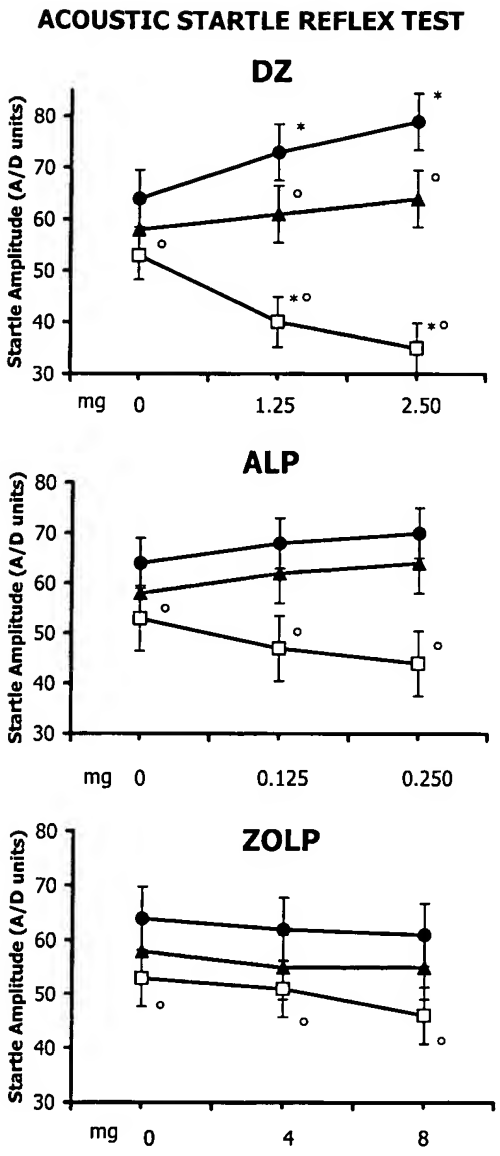

Fig. 2. Effects of prenatal exposure to vehicle, DZ, ALP and ZOLP on the ASR. Each value represents the mean±S.E.M. from 12 rats. *$P<0.05$ vs. respective controls; °$P<0.05$ vs. respective NH group. NH ●, SLH ▲, LLH □. NH, non-handled; SLH, short-lasting handled; LLH, long-lasting handled.

effect was weaker than the prenatal DZ NH, SLH and LLH ones. Rats exposed to ZOLP showed similar number of punished licks than controls. No differences were observed in the unpunished drinking levels in prenatally DZ, ALP and ZOLP-exposed rats, as compared to controls.

3.4. Open field test

The results of TDT in open field, performed across five experimental sessions, are shown in Fig. 3. Prenatally vehicle, DZ-, ALP- and ZOLP-exposed SLH rats typically displayed a similar mounting reduction in TDT during the sessions. Compared to controls, DZ- and ALP-exposed rats, respectively, displayed significant reduction and increase in TDT. Prenatal ZOLP did not modify TDT.

3.5. Operant behaviour test

The results of the lever pressing responses in the Skinner-box performed on five consecutive sessions are displayed in Fig. 3. Compared to controls, prenatally DZ-exposed SLH rats displayed a significant decrease in the number of RV 30 reinforced schedule lever pressing responses, while rats exposed to ALP showed a significant increase. Prenatal exposure to ZOLP did not change the number of emitted responses.

3.6. Sensitivity to picrotoxin

The number of convulsed prenatally vehicle-, DZ-, ALP- and ZOLP-exposed SLH rats after different doses of PTX (1.0; 2.5; 4.0 mg/kg i.p.) is displayed in Table 5. Compared to controls, prenatal exposure to DZ and ALP induced the appearance of convulsions in a greater number of rats when they were challenged with the highest doses of PTX. Prenatal exposure to ZOLP did not influence the effect of PTX.

Table 4
Effects of prenatal exposure to vehicle, DZ, ALP and ZOLP on unpunished (unpun.) and punished (pun.) licks on the VT in adult rat offspring

Prenatal treatment (mg/kg)	NH		SLH		LLH	
	Unpun.	Pun.	Unpun.	Pun.	Unpun.	Pun.
Vehicle	186±17	9±2	192±18	12±2	197±23	16±2°
Diazepam						
1.250	178±15	4±1*	200±16	6±2*	205±20	11±2*°
2.500	172±16	3±1*	205±15	5±1*	208±17	8±2*°
Alprazolam						
0.125	195±14	8±1	206±16	11±3	210±18	13±2°
0.250	193±18	5±1*	208±20	8±2*	205±21	11±2*°
Zolpidem						
4.0	192±14	10±2	200±21	12±1	196±24	15±2°
8.0	190±16	9±1	196±18	11±2	192±22	14±2°

Each value represents the mean±S.E.M. from 12 rats. *$P<0.05$ vs. respective controls; °$P<0.05$ vs. respective NH group.

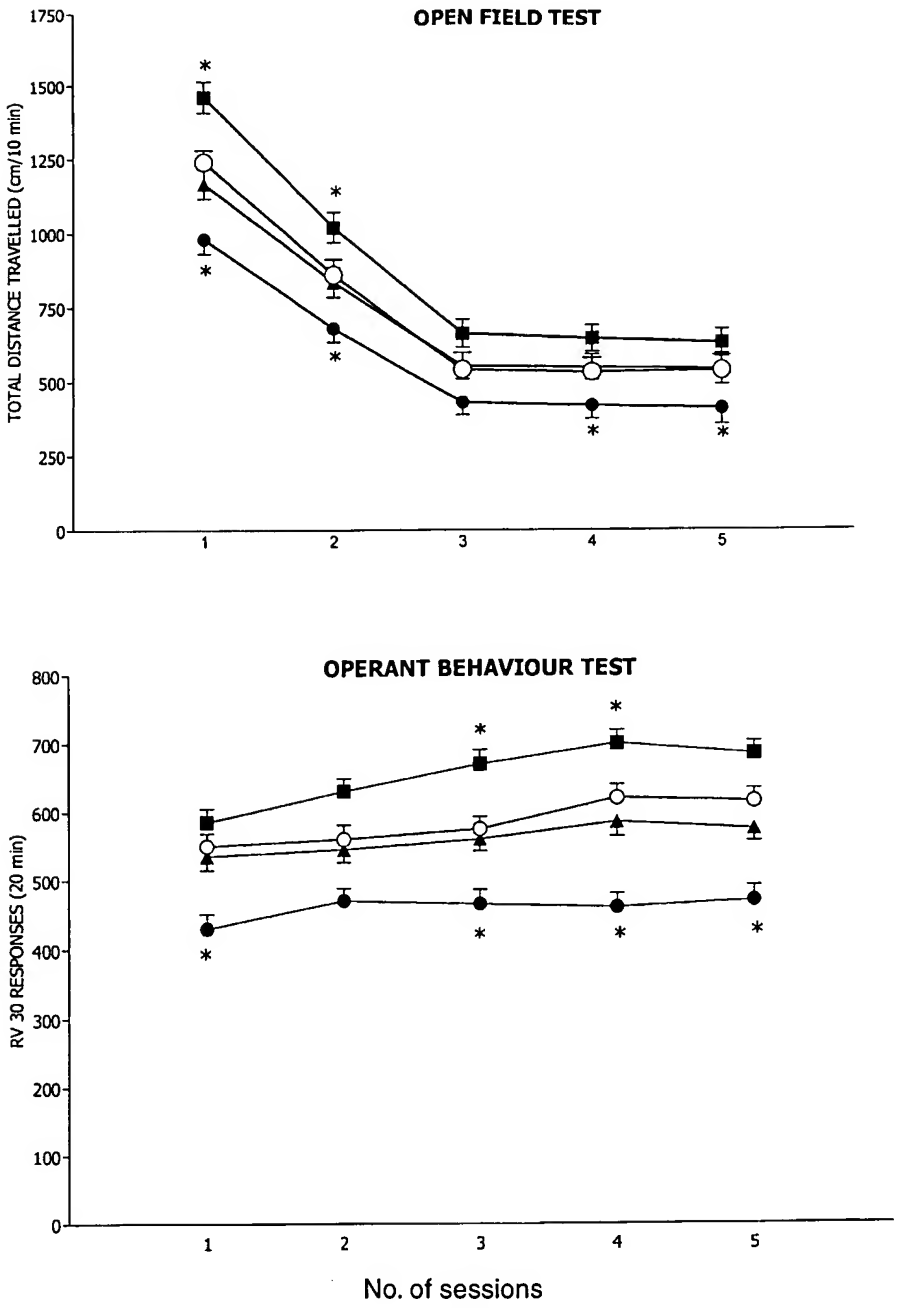

Fig. 3. Effects of prenatal exposure to vehicle ○, DZ ●, ALP ■ and ZOLP ▲ on the total distance travelled in the open field test and on total lever pressing responding in the Skinner box in SLH adult offspring. Each value represents the mean±S.E.M. from 16 rats for the open field test and from six rats for the Skinner box. *$P<0.05$ vs. prenatal exposure to vehicle.

Table 5
Effects of prenatal exposure to vehicle, DZ, ALP and ZOLP on the incidence of convulsions after acute treatment with PTX in SLH adult rat offspring

Prenatal treatment (mg/kg)	Picrotoxin (mg/kg)		
	1.0	2.5	4.0
Vehicle	–	1	3
Diazepam 2.50	2	6	10
Alprazolam 0.25	1	4	8
Zolpidem 8.0	–	2	4

Each value represents the number of rats showing tonic–clonic seizures out of 10 rats.

4. Discussion

The present study was aimed at investigating the effects of late prenatal exposure to different GABA/BDZ R agonists on behavioural stress reactivity in handling-naïve and handling-habituated adult male rat progeny. The results indicate that prenatal exposure to DZ and ALP is effective in influencing this behavioural pattern, while ZOLP is not. Furthermore, this study shows that long-lasting handling is able to overcompensate the changes induced by prenatal exposure to DZ and ALP in certain stress-evoked behavioural patterns.

The effects of a late prenatal exposure to DZ on the stress-induced responsiveness of the central GABA/BDZ R complex [38], suggest that this benzodiazepine can interfere with the organisation of the GABA system leading to a long-term decrease in the functional response of the GABA/BDZ R complex in the adult rat offspring [38,43]. Indeed in this research, prenatal exposure to DZ and ALP, but not to ZOLP, induced an increase in the seizure sensitivity of SLH rats to PTX, when compared to respective controls. The seizure sensitivity to PTX was studied in SLH rats exposed to DZ, ALP and ZOLP, because these rats showed a level of emotionality similar to vehicle-exposed rats. This condition allowed evaluation of the functional state of GABA/BDZ Rs as an index of the prenatal drug treatment, independently from variations in emotionality due to non-handling and long lasting handling procedure. The increase in seizure sensitivity to PTX is in agreement with previous data from our group indicating that rats prenatally exposed to DZ show a strong increase in the electrographic hippocampal response to PTX after intra locus coeruleus injection of this compound [13,15]. Thus, the increased sensitivity to PTX observed in prenatally DZ- and ALP-exposed rats is consistent with a reduction in the response of central GABA/BDZ Rs induced by the prenatal drug treatment.

When exposed to FST, NH DZ-exposed rats display a significant decrease in immobility time compared to NH vehicle-exposed rats. Conversely, higher values of immobility time are recorded in LLH DZ-exposed rats, when compared to the respective controls. NH and LLH rats exposed to ALP show similar, but less marked changes. In SLH rats, no differences were recorded between DZ-, ALP- and vehicle-treated rats.

Previous researchers have shown that in response to environmental challenges, i.e. forced swim, there is an increased response of GABA/BDZ Rs in the cerebral cortex of male rats [31,60,61]. This effect and the increase in immobility time in FST have been interpreted as an adaptive physiological response to this potentially dangerous situation [24,31,56,61]. Moreover, when rats are less stressed, they display decreased escape-oriented activity in the FST and a reduction in the stress-induced neurochemical and endocrine alterations [35]. On the other hand, at doses that decrease the anxiety-related behaviour and do not modify locomotor activity, acute DZ treatment increases immobility time in the FST (data not published); conversely, acute anxiogenic PTX [22] which reduces motor activity in open field, decreases immobility time [12,14]. Therefore, the lower values in immobility time observed in the FST in NH DZ- and ALP-exposed animals seem to reflect a higher sensitivity to the stress of the forced swim, with respect to NH vehicle-exposed animals. Short-lasting handling is able to prevent the effect exerted by prenatal DZ and ALP, as no differences are observed between vehicle- and drug-exposed rats. On the contrary, long-lasting handling increases immobility time in DZ- and ALP-exposed rats. This could mean that animals prenatally treated with the two drugs are more sensitive to handling procedure than vehicle-exposed rats and that LLH rats show lower values of emotionality when exposed to the forced swim, with respect to NH rats, either treated or not.

In the ASR test NH DZ-exposed rats display a significant increase in the peak amplitude of the response, while LLH DZ-exposed rats show a significant decrease compared to their respective controls. Prenatal exposure to ALP induces similar but less pronounced effects. Again, short-lasting handling is able to counteract the effects of the two drugs as no significant differences are observed between SLH vehicle- and DZ-, ALP-exposed rats.

The ASR, a reflexive movement occurring after a sudden exposure to a loud noise, represents a valid behavioural model to study the emotional response of the animals. An increase or a decrease in the amplitude of the ASR are ascribed to a rise or a reduction in emotionality, respectively [32]. Anxiogenic drugs increase the amplitude of the response, while anxiolytic drugs reduce it [32,36]. Thus, the results from the ASR appear to be consistent with the effects observed in the FST, confirming a higher sensitivity to aversive stimuli in handling-naïve rats and a lower sensitivity in LLH rats.

Indeed, neurochemical and behavioural differences exist in handling-naive and handling-habituated rats. In particular, significant differences have been reported in GABA/BDZ R expression. Decreases or increases in the GABA/BDZ R response related to increased or decreased anxiety-related behaviours have been observed in NH and LLH rats, respectively [17,21,61]. Moreover, in handling-naive animals, stress-evoked changes in neurochemical and behavioural patterns are similar to those exerted by anxiogenic drugs, and benzodiazepines produce consequences similar to handling habituation [9,23]. Therefore, the opposite effects in the FST and the ASR observed in NH and LLH rats prenatally exposed to DZ and ALP might respectively reflect a different response of GABA/BDZ Rs, as a result of non-handling and long-lasting handling procedure. The lack of differences between SLH DZ and ALP-exposed rats and SLH vehicle-exposed rats, would suggest that a short-lasting handling is able to correct the alterations in the stress-related behavioural responses induced by prenatal benzodiazepines.

Data from the VT lead to different conclusions. In fact, LLH DZ- and ALP-exposed rats lick less than the respective controls during the punished period. The same is true for NH and SLH DZ- and ALP-exposed animals. These results are not explicable on the basis of different baseline rates of drinking, because the animals do not differ in unpunished drinking levels. In the VT, the shock-induced inhibition of licking measures the anxiety-related behaviour [63]. In this study the inhibition of licking during the punished period is more evident in NH DZ- and ALP-exposed rats than the controls. Thus, it would seem that the perception of the stressful nature of the paradigm is more intense in DZ- and ALP-exposed rats than in vehicle-exposed rats. On the other hand, reduction in the functional

response of GABA/BDZ Rs, induced by prenatal benzodiazepine treatment, could increase pain sensitivity in rats making the animals more sensitive to the electric shock. This may explain why long-lasting handling attenuates the punished licking inhibition, but is not able to 'normalise' the altered behavioural pattern in the rats prenatally exposed to DZ and ALP.

The effects of the prenatal treatment with DZ, ALP and ZOLP on the OFT and the Skinner-box test were subsequently evaluated in SLH rats. Only SLH animals have been employed since NH rats could not be used as the execution of these tests requires a repeated manipulation of the animals. LLH rats have been excluded because long-lasting handling procedure produces in the animals effects on the emotional state, which interfere with the specific effect of prenatal DZ treatment on motor activity [15]. SLH animals then allowed us to rule out the involvement of emotionality in the evaluation of the effects of prenatal DZ and ALP in the motor activity, since the SLH DZ- and ALP-treated rat performance in FST and ASR was similar to controls.

Prenatal treatment with DZ induced a significant decrease in the locomotion and in the lever pressing responses in the Skinner-box, while prenatal treatment with ALP induced a significant increase compared to controls. The opposite effects induced by DZ and ALP on locomotion and on lever pressing responses are not unexpected. DZ and ALP have different pharmacological profile and induce different behavioural effects [4,16,25,62].

Prenatal exposure to ZOLP did not induce significant modifications in the behavioural models examined, although the doses employed in this study provoked sedative effects in pregnant rats. This could be explained by its receptor selectivity [19,42]. Indeed, ZOLP, a hypnotic imidazopyridine compound, facilitates GABA transmission acting on the BDZ ω1 receptor subtype [28,41]. In fact, it recognises preferentially those GABA-A R subtypes comprised of α1 subunit, acting therefore on a restricted number of receptors [55]. Moreover, in vivo binding studies demonstrate that ZOLP has a lower level of receptor occupancy than classic benzodiazepines [6]. These findings are in agreement with our previous research reporting the ineffectiveness of ZOLP in influencing the behavioural effects of drugs acting on GABA/BDZ Rs [14].

In summary, our results provide a comprehensive study of the effects of prenatal DZ, ALP and ZOLP exposure on rat behavioural reactivity in aversive situations, and of the influence of handling in modulating the anxiety-related behaviours. In particular, this study shows a convergence on behavioural stress reactivity and a differentiation in the effects of prenatal exposure to DZ and ALP on motor activity, while ZOLP is ineffective. Furthermore, our data indicate that a long-lasting handling is able to overcompensate for alterations in the stress-related behavioural responses induced by prenatal treatment with benzodiazepines. Therefore, handling habituation may be considered a useful strategy to enhance rat ability to cope with stress acting on the plasticity of the GABA/BDZ Rs, even following a benzodiazepine treatment administered during the ontogenetic period.

This study may have a clinical impact. Benzodiazepines are commonly used by pregnant women, intentionally or unintentionally for their anxiolytic, sedative and hypnotic properties. Although benzodiazepine use is probably not associated with an increase in the incidence of birth defects, our data suggest that benzodiazepines might induce in humans subtle developmental or behavioural alterations, which may be observed at a later time. On the contrary, ZOLP, at least on the behavioural models used, does not seem to induce any alterations and, therefore, it may be a preferable hypnotic in pregnant women.

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Genetic and Pharmacological Evidence of a Role for $GABA_B$ Receptors in the Modulation of Anxiety- and Antidepressant-Like Behavior

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Although there is much evidence for a role of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) in the pathophysiology of anxiety and depression, the role of $GABA_B$ receptors in behavioral processes related to these disorders has not yet been fully established. $GABA_B$ receptors are G-protein-coupled receptors, which act as functional heterodimers made up of $GABA_{B(1)}$ and $GABA_{B(2)}$ subunits. Using recently generated $GABA_{B(1)}{}^{-/-}$ mice, which lack functional $GABA_B$ receptors, and pharmacological tools we assessed the role of $GABA_B$ receptors in anxiety- and antidepressant-related behaviors. In the light–dark box, $GABA_{B(1)}{}^{-/-}$ mice were more anxious than their wild-type littermates (less time spent in the light; reduced number of transitions). $GABA_{B(1)}{}^{-/-}$ mice were also more anxious in the staircase test. Conversely, acute and chronic treatment with GS39783, a novel $GABA_B$ receptor positive modulator, decreased anxiety in the light–dark box and elevated zero maze tests for anxiety. On the other hand, $GABA_{B(1)}{}^{-/-}$ mice had decreased immobility (antidepressant-like behavior) in the forced swim test (FST). These behavioral effects are unrelated to alterations in locomotor activity. In confirmation of the genetic data, acute and chronic treatment with CGP56433A, a selective $GABA_B$ receptor antagonist, also decreased immobility in the FST, whereas GS39783 did not alter this behavior. Taken together, these data suggest that positive modulation of the $GABA_B$ receptor may serve as a novel therapeutic strategy for the development of anxiolytics, whereas $GABA_B$ receptor antagonism may serve as a basis for the generation of novel antidepressants.

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Keywords: GABA; depression; mood disorder; positive modulator; GS39783

INTRODUCTION

γ-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the brain and hence GABAergic neurotransmission regulates many physiological and psychological processes. There are two classes of GABA receptors: ionotropic $GABA_A$ receptors and metabotropic $GABA_B$ receptors. The $GABA_B$ receptor is a heterodimer made up of two subunits, $GABA_{B(1)}$ and $GABA_{B(2)}$, both necessary for $GABA_B$ receptors to be functionally active (Calver *et al*, 2002). Clinical and preclinical evidence strongly implicates GABAergic dysfunction in anxiety (Millan, 2003) and depression (Brambilla *et al*, 2003; Krystal *et al*, 2002); however, evidence for a specific role for $GABA_B$ receptors is unclear. Although $GABA_B$ receptors were first proposed to play a role in psychiatric disorders such as depression and anxiety over 20 years ago (Pilc and Lloyd, 1984), further progress in the field has been largely hampered by the lack of appropriate tools. The prototypical $GABA_B$ receptor agonist baclofen, although highly selective and clinically available for over 30 years for the treatment of spasticity, produces severe sedation and muscle relaxation, which confounds its widespread use as a tool in behavioral paradigms related to anxiety and depression.

Two recent developments have added innovative new tools to the armamentarium of researchers. Firstly, mice that lack the $GABA_{B(1)}$ subunit (Prosser *et al*, 2001; Queva *et al*, 2003; Schuler *et al*, 2001) have been generated. Secondly, with positive allosteric modulators, novel pharmacological tools for $GABA_B$ receptors have been characterized (Urwyler *et al*, 2001; Urwyler *et al*, 2003). These molecules enhance the action of GABA at the $GABA_B$ receptor and have little or no intrinsic agonistic efficacy on their own (Urwyler *et al*, 2001; Urwyler *et al*, 2003). Application of $GABA_B$ receptor positive modulators in the presence of an agonist shifts the concentration–response curve to the left, as the modulators increase the potency of GABA. In addition, the maximal efficacy of GABA is increased. Allosteric positive modulation of metabotropic

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receptors is a recently identified phenomenon, providing novel means for the pharmacological manipulation of G-protein-coupled receptors acting at a site apart from the orthosteric binding region of the receptor protein (Soudijn *et al*, 2002). Such properties suggest that allosteric modulators may offer a number of potential pharmacological improvements over the use of conventional agonists as has been demonstrated for modulators acting at ligand-gated ion channels (Costa, 1989). In the case of $GABA_A$ receptors, such modulation has been therapeutically utilized with the benzodiazepines, which amplify the action of the endogenous neurotransmitter GABA. Therefore, we hypothesized that $GABA_B$ receptor positive modulators will be superior drugs, devoid of the side-effect profile associated with full agonists such as baclofen.

Therefore, we have novel tools, $GABA_{B(1)}$ knockout mice and positive modulators, to better examine the role of $GABA_B$ receptors in behavioral paradigms relevant to anxiety and depression. In these studies, we investigated the behavioral effects of mice lacking $GABA_{B(1)}$ receptor subunit in animal models of anxiety and depression and provide evidence for a role of $GABA_B$ receptors in the modulation of anxiety- and depression-like behavior. To further substantiate these observations in anxiety and depression paradigms, we investigated the behavioral effects of acute and chronic treatment of the selective $GABA_B$ receptor positive modulator GS39783 and the previously identified $GABA_B$ receptor antagonist CGP56433A (Brebner *et al*, 2002; Froestl *et al*, 1995).

MATERIALS AND METHODS

Animals

The $GABA_{B(1)}$ knockout mice were generated on a BALB/c background as described previously (Schuler *et al*, 2001). Age- and sex-matched mice were used at an age of 3–8 months. Both male and female animals were used in all experiments in approximately equal numbers, with the exception of animals used in the forced swim test (FST) and tail suspension test where only females were used. There was no effect of gender on behaviors observed. In order to minimize the influence of strain effects, all pharmacological studies were carried out in male BALB/c mice (23–26 g), which were obtained from Iffa Credo, France. In a number of initial studies, heterozygous mice ($GABA_B^{+/-}$) were also used. No gene dosage effect was found in any of the behaviors analyzed with heterozygotes behaving similarly to knockouts. Housing was at room temperature, in a 12 h light/dark cycle with lights on at 0600. Food pellets and tap water were available *ad libitum*. All behavioral experiments were conducted during the light cycle. All animals were experimentally naïve unless otherwise noted. Experiments were subject to institutional review and conducted in accordance with the Veterinary Authority of Basel-Stadt, Switzerland.

Light–Dark Box

The light–dark box test was carried out essentially as described previously (Cryan *et al*, 2003b; Holmes *et al*, 2002). The apparatus consisted of a clear plexiglass cage ($44 \times 21 \times 21$ cm) separated into two compartments by a partition, which had a small opening (12×5 cm) at the floor level. The open compartment was open topped made of transparent plexiglass and brightly illuminated by a 60 W desk lamp overhead (approximately 1000 Lux). The smaller compartment was 14 cm long and made from black plexiglass. It was covered on top also by black plexiglass. Mice were individually placed in the center of the brightly lit compartment, facing away from the partition and allowed to explore freely the apparatus for 10 min. The apparatus was cleaned thoroughly between subjects. The number of light–dark transitions, time spent in the light compartment, and latency to enter dark were recorded by a trained observer, with transitions being the most reliable indicator of anxiety-like behavior in the test (Crawley and Davis, 1982; Holmes, 2001). Two separate cohorts of $GABA_{B(1)}$ mice were used to confirm the phenotype.

Staircase Test

The test was carried out essentially as described earlier (Cryan *et al*, 2003b; Simiand *et al*, 1984) and consists of placing an experimentally naïve mouse in an enclosed staircase with five steps made of gray plastic. Each step was 2.5 cm in height, 7.5 cm in length, and 11 cm in width. The apparatus was 45 cm in length with one end 12 cm and the other 25 cm in height. The number of steps climbed and rearings made in a 3-min period were observed. The step-climbing count was increased every time the animal moved from one step to another in the ascending direction. The apparatus was briefly wiped with a wet paper towel and dried between animals. Animals were moved to the testing room at least 1 h prior to testing. The test has been validated using different anxiolytics (Simiand *et al*, 1984; Pick *et al*, 1996; Weizman *et al*, 1999) and has been used to examine anxiety-related phenotypes in genetically modified animals (Cryan *et al*, 2003b; Salas *et al*, 2003). The number of steps climbed and the rearing behavior of the mice are recorded as measures of anxiety-related behavior.

Elevated Zero Maze

This test is similar to the more widely used elevated plus maze in that both tests rest upon similar naturalistic conflicts between the tendency to explore a novel environment and aversive properties of a novel brightly lit, open, and elevated area. However, whereas the elevated plus maze has a center area that is neither in the open or closed part of the arena, it can be difficult to interpret the level of anxiety of an animal if it stays in this central part. Indeed the $GABA_B$ agonist baclofen has been shown to promote time in the center of the plus maze (Dalvi and Rodgers, 1996). The zero maze has no central area, so the animal must be in either an open or a closed part of the arena. The apparatus was a 5.5-cm-wide circular track constructed of gray plexiglass with an inside diameter of 34 cm, a mid-track circumference of approximately 121 cm, and an elevation of 40 cm. It consisted of two open quadrants with a raised, 2 mm edge and two closed quadrants with walls 11 cm high. Mice were placed in one of the closed quadrants designated as the starting quadrant and were allowed to investigate the zero maze for a period of 5 min. During this time, an

observer scored mice on several anxiety-related variables as identified in previous studies (Shepherd *et al*, 1994; Tarantino *et al*, 2000). These included time spent in both open and closed quadrants, number of transitions between quadrants, latency to leave the dark quadrant, stretchings (elongated body posture with at least snout over open/closed divide) into open quadrant, rearings, grooming, head dips, and number of fecal boli in both open and closed areas.

Measurement of Locomotor Activity

Animals were placed in automated locomotor activity cages (31 cm length, 19 cm width, 16 cm height; TSE, Bad Homburg, Germany) and the distance traveled was measured by the number of horizontal beam-breaks as previously described (Spooren *et al*, 2000). Data were collected using a personal computer in 5 min intervals. In experiments involving $GABA_{B(1)}$ mice or chronic treatments, data were assessed in mice that were unhabituated to the apparatus. In order to detect any potential drug-induced hyperactivity, CGP56433A was administered to mice after 60 min habituation to the apparatus.

Forced Swim Test

FST was conducted as previously described (Cryan *et al*, 2001, 2003b). Briefly, mice were placed individually into plexiglass cylinders (24 cm tall × 21 cm in internal diameter) filled with water (23–25°C) to a depth of 15 cm. All test sessions were recorded by a video camera positioned directly above the cylinders. Videotapes were subsequently scored blind by a trained observer. The behavioral measure scored from videotape was the duration of immobility during the last 4 min of the 6 min test period as previously validated (Porsolt *et al*, 1978). A mouse was judged to be immobile when making only those movements necessary to keep its head above water.

Tail Suspension Test

The tail suspension test was carried out essentially as described previously (Cryan *et al*, 2003a, b; Steru *et al*, 1985), with the exception that an automated device was used to score immobility (BioSeb, Chaville, France). Mice were individually suspended by the tail to a metal hook (distance from floor = 18 cm) using adhesive tape (distance from tip of tail = 2 cm). Typically, mice demonstrated several escape-oriented behaviors interspersed with temporally increasing bouts of immobility. The computer recorded the number of seconds spent immobile over the entire 6 min period.

Drugs

Desipramine and chlordiazepoxide were obtained from Sigma (St Louis, MO). Fluoxetine, L-baclofen, GS39783 (*N,N'*-dicyclopentyl-2-methylsulfanyl-5-nitro-pyrimidine-4, 6-diamine), and CGP56433A (3-{1(*S*)-[3-(cyclohexyl-methyl)hydroxyphosphinyl)2(*S*)hydroxypropylamino]nethyl} benzoic acid) were synthesized in-house. All drugs were made up fresh prior to use and administered orally in a suspension of 0.5% methylcellulose at a concentration of 10 ml/kg. In the case of chronic studies, animals were injected in the afternoon (1400–1800) for 21 days and tested (either in light–dark box or in FST) on the morning following last injection. They were again injected immediately after the initial test and for the consecutive day, locomotor activity testing was carried out approximately 24 h following this last injection. Doses for chronic studies were selected from previous studies showing robust effects at these doses (Borsini *et al*, 2002) or the dose–response studies of acute administration of the compounds (data presented in these studies).

Statistics

All data were analyzed using the appropriate within-subject, and mixed-design ANOVAS or Student's *t*-test (in the case of comparisons between just two groups of animals) followed by, where appropriate, Fisher's *post hoc* tests. The level of significance was set at $P<0.05$.

RESULTS

Impact of Targeted $GABA_{B(1)}$ Receptor Subunit Deletion on Anxiety-Related Behavior

Light–dark box. Upon being placed in the light side of the apparatus, freezing behavior was observed in 30% of the $GABA_{B(1)}^{-/-}$ mice but none of the wild type. As shown in Figure 1, $GABA_{B(1)}^{-/-}$ mice displayed marked increases in anxiety-related behaviors in the light–dark box paradigm compared with wild-type ($GABA_{B(1)}^{+/+}$) or heterozygous ($GABA_{B(1)}^{-/+}$) mice. ANOVA revealed a significant effect of genotype on the time spent in the light compartments ($F(2,45)=11.02$, $P=0.001$) and on the number of transitions ($F(2,45)=4.39$, $P=0.018$). Further, there was a genotype influence on the latency to enter the dark compartment ($F(2,45)=4.86$, $P=0.012$.). *Post hoc* analysis revealed that $GABA_{B(1)}^{-/-}$ mice exhibited a decrease of the latency to enter the dark compartment compared to wild-type heterozygous mice. $GABA_{B(1)}^{-/-}$ mice showed a significant decrease in the time spent in the light compartment compared to heterozygote or wild-type mice (Figure 1b) and exhibited significantly fewer light–dark transitions (Figure 1a). This latter parameter was the most reliable indicator of anxiety in the light–dark box test. Heterozygote mice behaved in the same manner as wild-type mice in all parameters in this test. Altogether, these effects are indicative of an increased anxiety in $GABA_{B(1)}^{-/-}$ mice. In order to confirm the reliability of the phenotype, a second cohort of animals were tested in the light–dark box. These $GABA_{B(1)}^{-/-}$ mice had both qualitatively and quantitatively the same (anxious) phenotype (data not shown).

Staircase test. In the staircase test, another paradigm for assessing anxiety-related behaviors, $GABA_{B(1)}^{-/-}$ mice had lower number of rearings than wild-type and heterozygote mice ($F(2,45)=23.15$, $P=0.001$) (Figure 3b). In addition, the number of steps climbed by $GABA_{B(1)}^{-/-}$ mice was decreased compared to wild-type and heterozygote mice ($F(2,45)=52.61$, $P=0.001$) (Figure 3a). This lack of exploration in the test was associated with a substantial amount of freezing behavior.

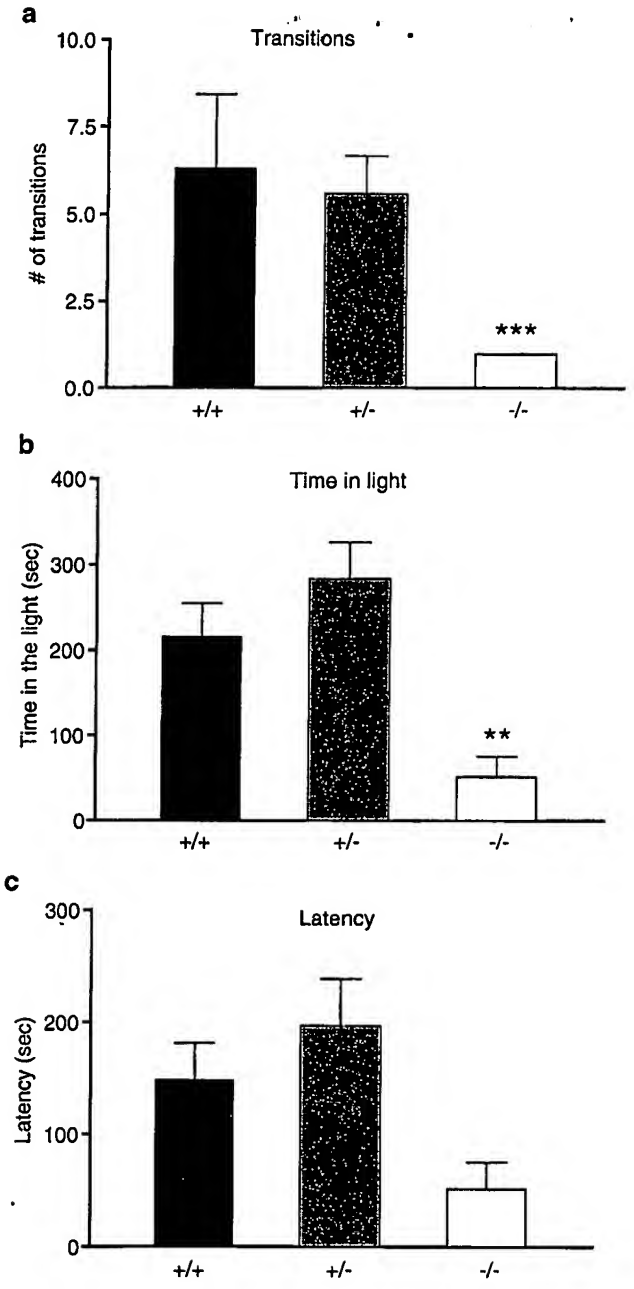

Figure 1 Increased anxiety in $GABA_{B(1)}$-deficient mice in the light–dark box. (a) $GABA_{B(1)}^{-/-}$ mice had a marked decrease of transitions between light and dark compartments compared with heterozygote or wild-type mice. (b) $GABA_{B(1)}^{-/-}$ mice spent less time in the light compartment in comparison to heterozygous or wild-type mice. (c) $GABA_{B(1)}^{-/-}$ mice ($n=16$) exhibited a decrease in latency to enter the dark compartment, compared to heterozygous ($n=16$), but not compared to wild-type mice ($n=16$). All bars represent mean values, with vertical lines indicating one SEM. *, **, ***Groups that differed significantly compared to wild-type mice ($P<0.05$, <0.01, and <0.001, respectively).

In summary, both the behavior in the light–dark test and staircase tests score demonstrate an increased level of anxiety in $GABA_{B(1)}^{-/-}$ mice.

Elevated zero maze. No functional data were obtained from examining the behavioral response of $GABA_{B(1)}^{-/-}$ mice in the elevated zero maze due to the fact that all of the $GABA_{B(1)}^{-/-}$ mice actively jumped off the maze. The

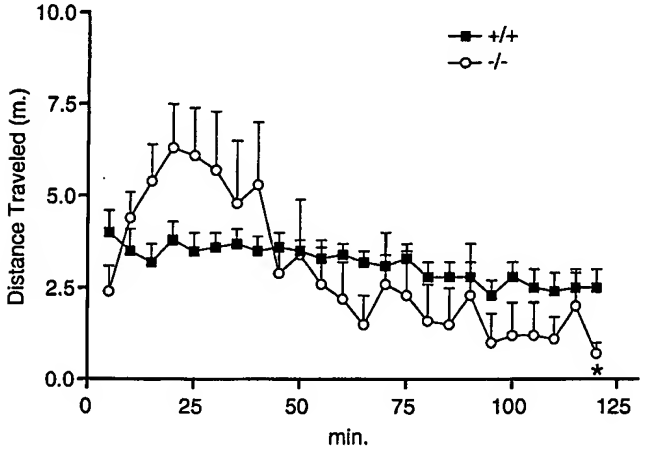

Figure 2 Effect of $GABA_{B(1)}$ deletion on locomotor activity in naïve mice. No significant effect of genotype was seen; however, three distinct phases of activity were observed in $GABA_{B(1)}^{-/-}$ mice compared with wild type: hypoactivity followed by a hyperactive response followed by rebound hypoactivity. $n=20$ per genotype group. All bars represent mean values, with vertical lines indicating one SEM. *Groups that differed significantly compared to wild-type mice ($P<0.05$).

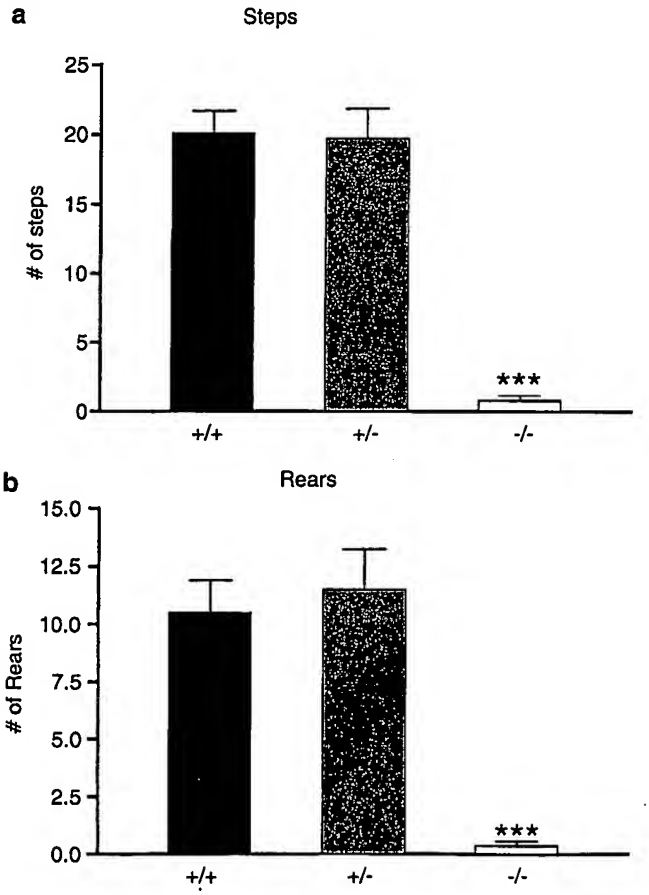

Figure 3 Increased anxiety in $GABA_{B(1)}^{-/-}$ mice in the staircase test. $GABA_{B(1)}^{-/-}$ mice ($n=16$) exhibited a decrease in the steps climbed compared to heterozygous ($n=16$) and wild-type ($n=16$) mice. (b) $GABA_{B(1)}^{-/-}$ mice had significantly less rearing events compared to heterozygous or wild-type mice. All bars represent mean values, with vertical lines indicating one SEM. ***Groups that differed significantly compared to wild-type mice ($P<0.001$).

reasons for this increased flight response are likely to reflect an increase in anxiety/panic-like behavior as opposed to lack of motor coordination as evidenced by absence of motor deficits in rotarod tests (Schuler *et al*, 2001; C Mombereau and JF Cryan unpublished observations). Further, similar flight reactions from an unstable elevated maze have been recently characterized as a novel model of panic/anxiety in rodents (Jones *et al*, 2002a, b; King, 1999a, b). Additionally, such an ethological response has also been demonstrated in the wild house mouse (*Mus musculus*) in the elevated plus maze (Holmes *et al*, 2000).

Locomotor activity tests in $GABA_{B(1)}^{-/-}$ mice. As shown in Figure 2, the locomotor activity of $GABA_{B(1)}^{-/-}$ mice is complex and can be divided into three parts: a short 'low-activity' pattern' (0–5 min), a 'rebound' pattern associated with a large increase of locomotor activity (10–45 min), and finally a pattern of hypoactivity (45–120 min). ANOVA revealed no effect of genotype on locomotor activity ($F(1,38)=0.053$, $P=0.819$), and there was a significant genotype × time interaction ($F(23,874)=3.221$, $P=0.001$).

Effects of a $GABA_B$ Receptor Positive Modulator on Anxiety-Related Behavior

Given the anxious phenotype of $GABA_B$ receptor knockout mice, we hypothesized that activation of the $GABA_B$ receptor would reduce anxiety. Hence we tested the effects of a novel $GABA_B$ receptor positive modulator GS39783 (Urwyler *et al*, 2003) in animal models of anxiety.

Light–dark box test. As shown in Figure 4, ANOVA indicated an effect of drug treatment on the number of transitions between dark and light compartments ($F(4,45)=10.06$, $P=0.001$). *Post hoc* analysis revealed that GS39783 (0.3–30 mg/kg, p.o.) and the benzodiazepine chlordiazepoxide (10 mg/kg, p.o.) increased the number of transitions. Treatment with GS39783 or chlordiazepoxide 1 h prior to testing failed to influence the latency to enter the dark chamber but increased the time spent in the light compartment ($F(4,45)=9.30$, $P=0.001$). *Post hoc* analysis indicated a significant effect of both chlordiazepoxide and GS39783 (only at the highest dose tested—30 mg/kg). These effects are not due to any confounding effect of GS39783 on locomotor activity as acute administration of GS39783 is devoid of any effects on locomotor activity (JF Cryan and WP Spooren, unpublished observations). It is of interest that the basal levels of anxiety in the light–dark test in Figure 4 are considerably different from those in Figure 1. The reason for this may lie in the fact that these mice are purchased from Iffa Credo and those in Figure 1 are wild-type BALB/c mice, which were housed with their more anxious littermates.

In an attempt to assess the effects of chronic administration of the positive modulator on anxiety-like behavior, we tested GS39783 in addition to CGP56433A (a selective $GABA_B$ receptor antagonist) and the antidepressants fluoxetine and desipramine in the light–dark box (20–24 h following last treatment). ANOVA revealed an effect of chronic drug treatment on the time spent in the light side of the arena ($F(4,55)=2.573$, $P=0.04$) and the number of

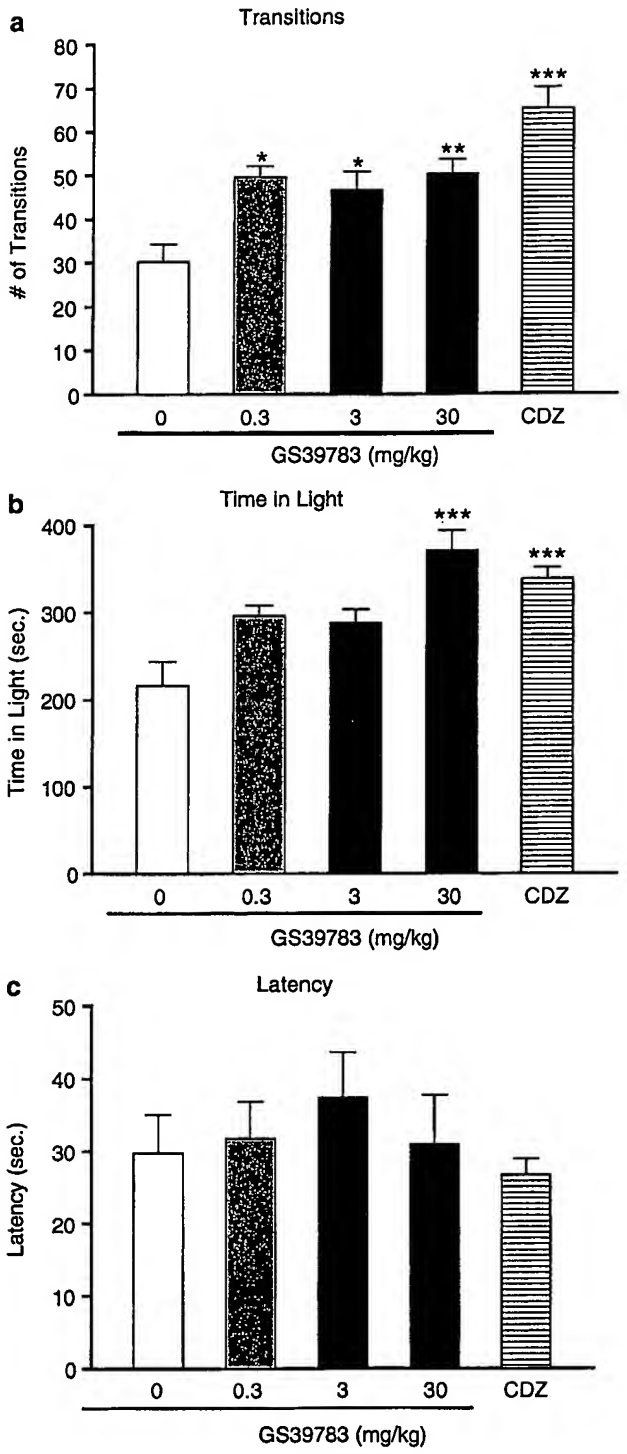

Figure 4 Anxiolytic effects of acute treatment with the $GABA_B$ receptor positive modulator GS39783 in the light–dark test. Effects of acute $GABA_B$ positive modulator treatment (doses: 0, 0.3, 3, or 30 mg/kg, p.o.) and chlordiazepoxide (CDZ, 10 mg/kg, p.o.) on (a) the number of transitions between light and dark compartments during the test, (b) the time spent in the light compartment, and (c) the latency to enter the dark compartment. $n=10$ per treatment group. All bars represent mean values, with vertical lines indicating one SEM. *,**,***Groups that differed significantly compared to vehicle-treated mice ($P<0.05$, <0.01, and <0.001, respectively).

transitions between the light and the dark sides ($F(4,55)=2.637$, $P=0.04$), but had no effect on the latency to enter the dark compartment (Figure 5a). *Post hoc* analysis revealed that GS39783 was the only compound tested to modify significantly the number of transitions (Figure 5a) and the time spent in the light side of the arena (data not shown). Taken together, these results indicate a potential anxiolytic effect of acute and chronic GS39783 treatment. As shown in Figure 5b, these effects are not due to any confounding effect of GS39783 on locomotor activity, as chronic administration of GS39783 did not affect locomotor activity ($F(4,53)=0.9289$, $P=0.4543$). It is of interest that the basal levels of anxiety in the light–dark test in Figure 5 are considerably different from those in Figure 4. The reason for this may lie in the fact that although all mice are purchased from Iffa Credo, those in Figure 5 have been handled and injected daily for 21 days and this stress has been shown to influence anxiety-like behavior in mice (Lapin 1995).

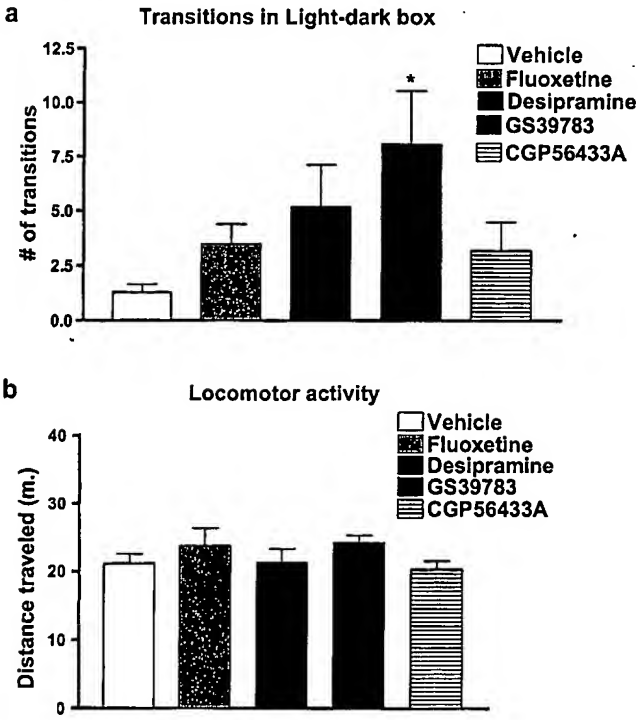

Figure 5 Chronic treatment with the $GABA_B$ receptor positive modulator reveals anxiolytic effects in the light–dark box test. Chronic treatment (21 days) with $GABA_B$ receptor positive modulator GS398783 (10 mg/kg, p.o., once daily) significantly increased (a) the number of transitions between light and dark compartments during the test, whereas fluoxetine (10 mg/kg, p.o., once daily), desipramine (15 mg/kg, p.o., once daily), and the $GABA_B$ receptor antagonist (3 mg/kg, p.o., once daily) were without effect. $n=12$ per treatment group. All bars represent mean values, with vertical lines indicating one SEM. *Groups that differed significantly compared to vehicle-treated mice ($P<0.05$). (b) Locomotor activity in a novel environment following chronic (23 days) administration of the $GABA_B$ receptor positive modulator (10 mg/kg, p.o.), fluoxetine (10 mg/kg, p.o.), desipramine (15 mg/kg, p.o.), and $GABA_B$ receptor antagonist (3 mg/kg, p.o.). Testing was carried out for 30 min 24 h following last dose in the same animals previously tested in the light–dark box. None of the treatments altered locomotor activity, indicating that the effects of GS39783 in the light–dark box are not due to any secondary stimulant effect. $n=12$ per treatment group. All bars represent mean values, with vertical lines indicating one SEM.

Elevated zero maze. To further confirm the anxiolytic effects of GS39783, we tested it in comparison with chlordiazepoxide in the elevated zero maze in BALB/c mice, the background strain on to which $GABA_{B(1)}{}^{-/-}$ mice were generated. ANOVA revealed that drug treatment decreased the latency to enter the open sides of the maze ($F(4,55)=3.192$, $P=0.020$), the number of stretched-attend postures ($F(4,55)=13.16$, $P<0.0001$) and increased the time spent in the open side of maze ($F(4,55)=3.932$, $P=0.007$), increased the number of head dips ($F(4,55)=6.995$, $P<0.00001$), number of rearings ($F(4,55)=8.233$, $P<0.0001$), and the number of line crossings ($F(4,55)=33.76$, $P<0.0001$). *Post hoc* analysis revealed that chlordiazepoxide (10 mg/kg p.o.) significantly affected all parameters tested, whereas GS39783 treatment reduced the latency to enter the open side at the highest dose tested (30 mg/kg, p.o.; $P<0.05$) (Figure 6a), and at doses of 3–30 mg/kg reduced the number of stretch-attend postures (Figure 6c) only. There was a trend toward GS39783 increasing the time in the open parts of arena, which failed to reach the level of significance (Figure 6b). GS39783 failed to affect the number of head dips, number of rearings, and the number of line crossings at any dose tested (data not shown). Taken together, these data further suggest an anxiolytic effect of GS39783, although the magnitude of the effects in this test are much less robust compared with that induced by benzodiazepine anxiolytics.

Impact of Targeted Deletion of $GABA_{B(1)}$ Receptor on Depressive-Related Behaviors

Forced swim test. The FST is the most widely used tool for assessing depression and antidepressant-related phenotypes in genetically altered mice (Cryan *et al*, 2002; Cryan and Mombereau, 2004; Porsolt, 2000); hence we examined the effects of mice with a targeted deletion of the $GABA_{B(1)}$ receptor subunit on behavior in this test. As shown in Figure 7a, there was a significant effect of genotype on immobility time in the FST (*t*-test, $P=0.012$). $GABA_{B(1)}{}^{-/-}$ mice had a significantly lower immobility time as compared to wild-type control mice. The magnitude of reduced immobility of the $GABA_{B(1)}{}^{-/-}$ mice in this test is similar to that we and others have reported for a variety of antidepressants, including selective monoamine reuptake or oxidase inhibitors (Cryan *et al*, 2001; Lucki *et al*, 2001; Porsolt *et al*, 1978). It is noteworthy that there was no observable occurrence of seizures or altered motor patterns in animals subsequent to being submerged in water.

Tail suspension test. We also tested the animals in the tail suspension test, another well-validated model for assessing depression-related behavior in mice (Steru *et al*, 1985). Further confirming accumulating evidence, that both tests rely on different neurochemical substrates to mediate their behavioral effects, deletion of $GABA_{B(1)}$ receptor subunit failed to affect the immobility score in this test (*t*-test, $P=0.710$) (Figure 7b). There was no observable occurrence of seizures or altered motor patterns in animals subsequent to being suspended by the tail. Further, no tail climbing was observed as has been reported with other background strains of mice (Mayorga and Lucki, 2001).

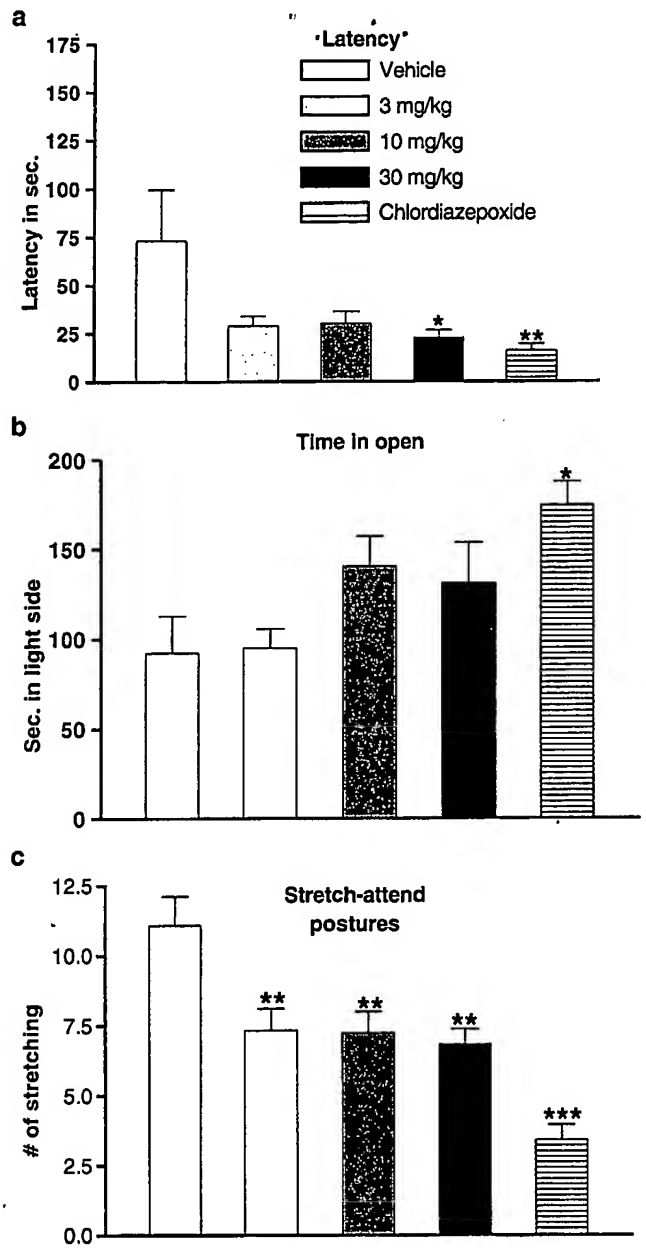

Figure 6 Effects of acute treatment with GS39783 on anxiety behavior in the elevated zero maze test. Both the acute $GABA_B$ positive modulator GS39783 and chlordiazepoxide (10 mg/kg, p.o.) affected (a) the latency to enter the open side of the maze and (c) the number of stretched-attend postures. However, only chlordiazepoxide significantly increased the time spent in the open quadrants of the maze (b). $n = 12$ per treatment group. All bars represent mean values, with vertical lines indicating one SEM. *,**,***Groups that differed significantly compared to vehicle-treated mice ($P<0.05$, <0.01, and <0.001, respectively).

Locomotor activity tests in $GABA_{B(1)}{}^{-/-}$ mice. In order to address the issue of whether the behavioral effects of $GABA_{B(1)}{}^{-/-}$ mice seen in the FST are related to potential hyperactivity, we analyzed the locomotor pattern. In a novel environment, the locomotor activity of the same mice that had previously undergone the FST was recorded over a period of 30 min. Repeated measures ANOVA revealed a clear impact of the targeted deletion of $GABA_{B(1)}$ receptor subunit on locomotor activity ($F(1,29) = 9.9$, $P = 0.001$). As

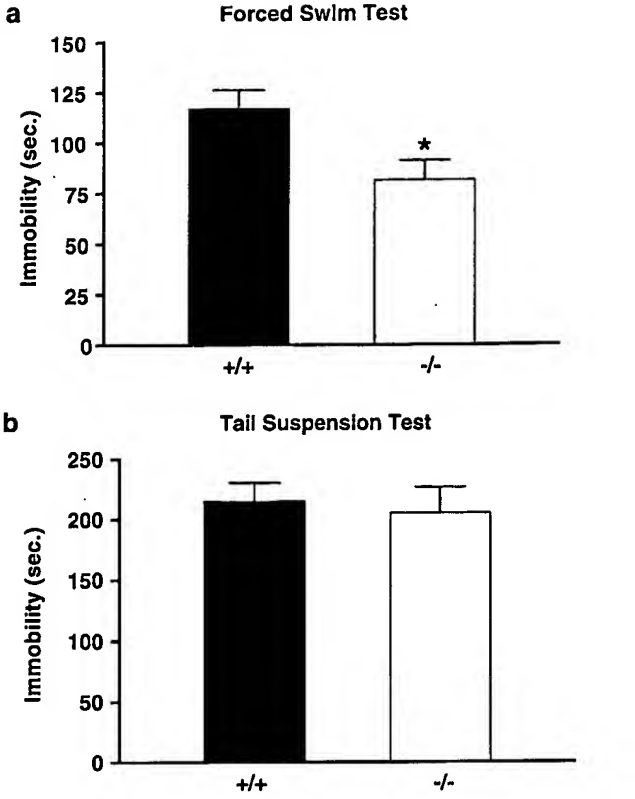

Figure 7 Antidepressant-like behavior in $GABA_{B(1)}{}^{-/-}$ mice. (a) $GABA_{B(1)}{}^{-/-}$ mice ($n = 16$) had a much lower immobility score than wild type ($n = 16$) in the mouse FST, which indicates an antidepressant-like effect. (b) $GABA_{B(1)}{}^{-/-}$ mice ($n = 15$) exhibited no difference in immobility compared to wild-type mice ($n = 16$) in the mouse tail suspension test. All bars represent mean values, with vertical lines indicating one SEM. *Groups that differed significantly compared to wild-type mice ($P<0.05$).

shown in Figure 8a, $GABA_{B(1)}{}^{-/-}$ mice exhibited a lower horizontal activity compared to wild-type mice during the first 20 min of the trial. This reduction of locomotor activity during the first minutes of trial could translate into a deficit in habituation to a novel environment in $GABA_{B(1)}{}^{-/-}$ mice and/or to an increased freezing behavior.

Correlations were also made between activity in the FST and the first 10 min in the novel locomotor activity chambers. Similar correlations were made with data obtained in the tail suspension test. As shown in Figure 8b, there was no correlation between locomotor activity (distance traveled) and immobility in FST in wild-type mice ($R = 0.349$, $P = $ NS) as well as in $GABA_{B(1)}{}^{-/-}$ mice ($R = 0.008$, $P = $ NS). These results suggest an absence of a stimulant effect as a result of $GABA_{B(1)}$ deletion. Additionally, no correlation was observed between immobility in the tail suspension test and locomotor activity in a novel environment (data not shown).

Effect of a $GABA_B$ Receptor Antagonist on Depressive-Related Behavior

Acute studies with CGP56433A. To test whether the antidepressant-like effect due to genetic deletion of the $GABA_{B(1)}$ receptor subunit could be recapitulated following pharmacological antagonism, we tested the highly selective and potent $GABA_B$ receptor antagonist CGP56433A in the

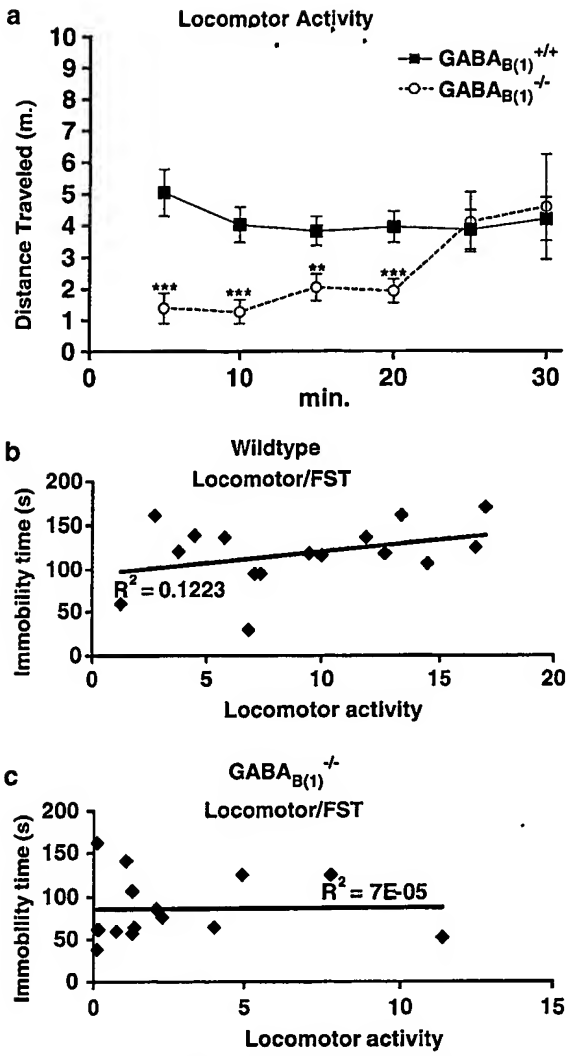

Figure 8 Effect of $GABA_{B(1)}$ deletion on locomotor activity in mice pretested with FST: deficits in habituation and lack of correlation with FST. (a) $GABA_{B(1)}^{-/-}$ mice ($n=15$) had a much lower locomotor activity score than wild-type mice ($n=16$) during the first 20 min of the 30 min trial. There was no consistent correlations between immobility score in the FST and locomotor activity score in wild-type mice (b) and $GABA_{B(1)}^{-/-}$ mice (c). All bars represent mean values, with vertical lines indicating one SEM. **, ***Groups that differed significantly compared to wild-type mice ($P<0.01$ and <0.001, respectively).

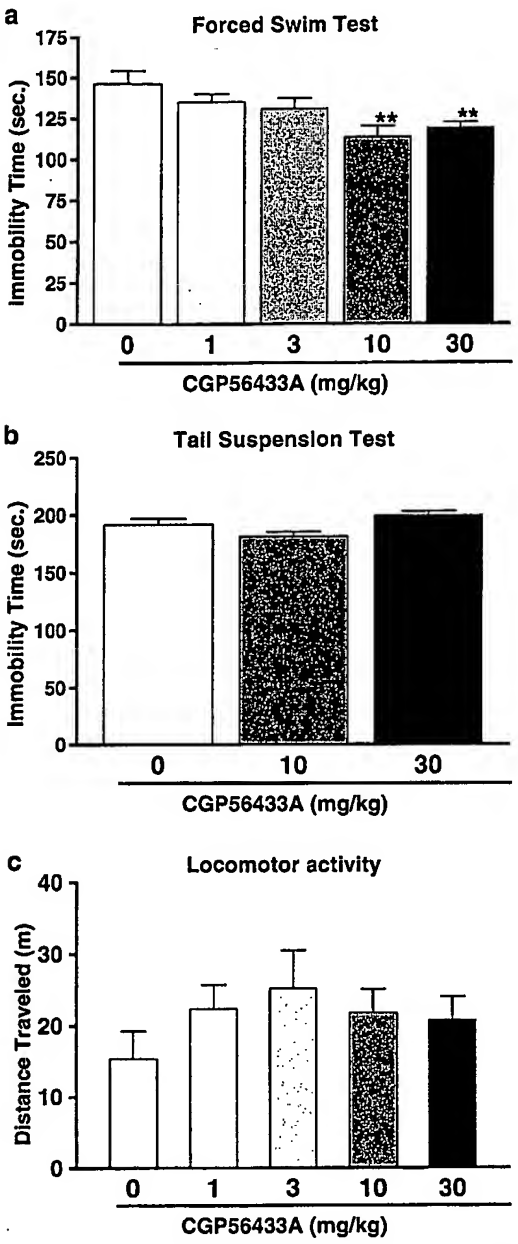

Figure 9 Acute treatment with CGP56433A reduces immobility in FST but not TST. (a) Effect of CGP56433A treatment (doses: 1, 3, 10, and 30 mg/kg, p.o.) on immobility time in FST. $n=10$–12 per treatment group. (b) Effect of CGP56433A treatment (doses: 0, 10, and 30 mg/kg, p.o.) on immobility time in the tail suspension test. $n=10$ per treatment group. (c) Effect of CGP56433A treatment (doses: 1, 3, 10, and 30 mg/kg, p.o.) on locomotor activity (60 min) in mice that were habituated (for 60 min) to the novel environment. $n=12$ per treatment group. All bars represent mean values, with vertical lines indicating one SEM. **Groups that differed significantly compared to vehicle-treated mice ($P<0.01$).

FST. As shown in Figure 9a, acute administration of CGP56433A affected immobility time in the FST ($F(4,53)=4.56$, $P=0.003$). *Post hoc* analysis revealed that CGP56433A (10 and 30 mg/kg) produced a significant decrease in immobility.

Further, we tested CGP56433A in the TST also. As shown in Figure 9b, CGP56433A failed to alter immobility in the test ($F(2,27)=0.24$, $P=0.791$), thus replicating the profile of genetic antagonism. Of note, CGP56433A failed to influence locomotor activity in habituated mice significantly (Figure 9b). These data exclude any potential stimulant effect of CGP56433A contributing to behavior in the FST.

Chronic studies. As shown in Figure 10, animals administrated chronically (21 days) with both CGP56433A (3 mg/kg, p.o., once daily) and desipramine (10 mg/kg, p.o., once daily) reduced immobility times in the FST whereas GS39783 was without any effect ($F(3,44)=7.966$, $P=0.001$).

DISCUSSION

In these studies, we sought to combine pharmacological and genetic approaches to obtain converging information on the

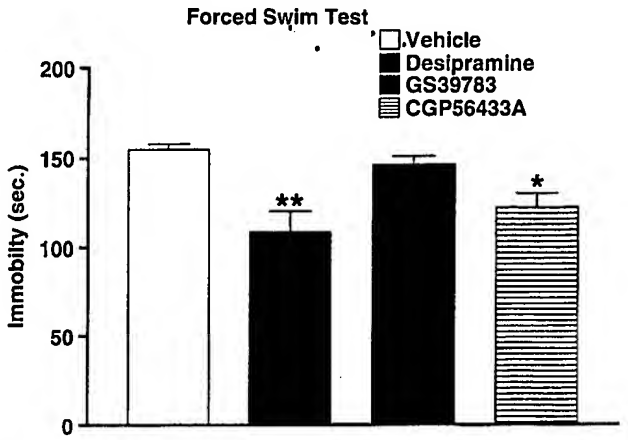

Figure 10 Chronic treatment with CGP56433A and desipramine reduces immobility in the FST. Effects of chronic treatment (21 days) with the $GABA_B$ antagonist CGP56433A (3 mg/kg, p.o.), desipramine (15 mg/kg, p.o.), and the $GABA_B$ positive modulator GS39783 (10 mg/kg, p.o.) on immobility time in the FST. $n = 12$ per treatment group. All bars represent mean values, with vertical lines indicating one SEM. *,**Groups that differed significantly compared to vehicle-treated mice ($P<0.05$ and <0.01, respectively).

function of $GABA_B$ receptors in behavioral processes. Using this dual approach, we demonstrate that through differential pharmacological manipulation of $GABA_B$ receptors, one can modify behaviors relevant to anxiety and depression. Deletion of $GABA_{B(1)}$ receptor subunit results in a more anxious phenotype in mice and an increased resistance to stress-induced behavioral despair. Congruent with these data, activation of $GABA_B$ receptors results in anxiolysis, whereas treatment with a $GABA_B$ receptor antagonist results in antidepressant-like effects in animal models. Given the complex overt behavioral phenotype of $GABA_{B(1)}^{-/-}$ mice, which includes a high propensity for spontaneous epileptic seizures, hyperalgesia, and amnesia (Schuler *et al*, 2001), it was important to combine both genetic and pharmacological approaches. Together, these studies clearly demonstrate that $GABA_B$ receptors play a role in the modulation of behaviors relevant to anxiety and depression.

Using the light–dark box, one of the most widely used tests for assessing anxiety-related behavior in rodents (Holmes, 2001), we clearly show that $GABA_{B(1)}^{-/-}$ mice are more anxious than their wild-type counterparts (Figure 1). Complimentary data were also found in the staircase anxiety test, where $GABA_{B(1)}^{-/-}$ mice had a substantial increase in freezing behavior and failed to explore the elevated platform compared to wild-type animals (Figure 3). It should be noted that this increase in anxiety-related behaviors is robust and not masked by the already high anxiety of the parental strain. In a variety of paradigms, it has been shown that BALB/c mice exhibit increased anxiety-related behaviors compared to other inbred strains of mice (Belzung and Griebel, 2001). The use of mice on this background strain was essential for the generation of $GABA_B$-related knockout animals, as mice on other background strains died very prematurely (Prosser *et al*, 2001; Queva *et al*, 2003). Interestingly, unlike genetic deletion, chronic pharmacological antagonism of $GABA_B$ receptors with CGP56433A failed to alter anxiety-related behavior in the light–dark box (Figure 5). This indicates that loss of the receptor during development may be critical for the increased anxiety phenotype to be unveiled; indeed using conditional knockout technology, such an assertion has recently been ascertained for the $5\text{-}HT_{1A}$ receptor (Gross *et al*, 2002). It is unlikely that the increased anxiety-like behavior is due to motor failure in the animals. Although $GABA_{B(1)}^{-/-}$ mice have less activity in locomotor chambers, their activity increases over time as they habituate to the environment (see Figures 2 and 8).

Given that $GABA_{B(1)}^{-/-}$ mice have elevated anxiety-like behavior, we hypothesized that by activating $GABA_B$ receptors we would be able to decrease anxiousness in normal animals placed in an aversive environment. Following acute administration of the recently identified $GABA_B$ receptor positive modulator GS37983 (Urwyler *et al*, 2003), animals displayed reduced anxiety in the light–dark box test (Figure 4) and elevated zero maze (Figure 6). Further, the anxiolytic effects of GS39783 were also observed following chronic treatment (Figure 5). Being a positive modulator, GS37983 is potentially advantageous over full $GABA_B$ agonists, which potentially engenders it more amenable for use *in vivo*. The major side effects associated with full agonists include sedation, muscle relaxation, hypothermia, and cognitive impairing effects.

Previous data investigating $GABA_B$ mechanisms in anxiety are limited and rather variable. This is largely because investigators relied on using the prototypical full $GABA_B$ receptor agonist baclofen for such analysis. Baclofen has a narrow efficacy window before confounding side effects are manifested in anxiety paradigms (Dalvi and Rodgers, 1996). That said, baclofen has demonstrated anxiolytic-like effects in a number of tests. It reduced separation induced calling by mouse pups (Nastiti *et al*, 1991) and enhanced punished drinking in rats (Ketelaars *et al*, 1988; Shephard *et al*, 1992) and had an anxiolytic-like response to novelty in a T-Maze (Quintero *et al*, 1985). Further, baclofen also reversed the anxiogenic response induced by withdrawal from chronic diazepam or alcohol treatment (Andrews and File, 1993; File *et al*, 1991; File *et al*, 1992). Clinically, baclofen reversed the anxiety associated with alcohol withdrawal (Addolorato *et al*, 2002) and post-traumatic stress (Drake *et al*, 2003). Thus our data suggest that $GABA_B$ receptor positive modulators may be a novel class of anxiolytic agents devoid of side effects associated with baclofen or benzodiazepines.

The mouse FST is the most widely used experimental paradigm for detecting antidepressant activity and to assess alterations in depression-like behavior in genetically modified animals (Borsini and Meli, 1988; Cryan *et al*, 2002; Cryan and Mombereau, 2004). The behavioral responses in the FST are thought to comprise a coping strategy (Thierry *et al*, 1984) in which immobility behaviors represent the psychological concept of 'entrapment' described in clinical depression (Dixon, 1998; Gilbert and Allan, 1998; Lucki, 2001). Here we demonstrate that $GABA_{B(1)}^{-/-}$ mice have an antidepressant-like effect in the FST as indicated by significantly lower immobility than their wild-type controls. This effect is not due to hyperactivity *per se*, as a reduced locomotor response was observed in the very same mice after being placed in a novel locomotor activity chamber, with activity increasing over

time. This is compatible with the anxious phenotype of $GABA_{B(1)}^{-/-}$ mice and suggests that they are more fearful upon being placed in a novel environment. In opposition to normal habituation responses in a novel environment, locomotor activity in $GABA_{B(1)}^{-/-}$ mice slowly increased with time, indicating a disinhibition of their initial anxiety. Further, there was no correlation between activity in the FST and that in the locomotor activity apparatus (Figure 8). This initial hypoactivity was unrelated to prior exposure to swim stress or age, as it was also evident (although not as pronounced) in experimentally naïve mice (Figure 2). However, at later time points, these animals became somewhat more active than wild-type controls, which is in accordance with previous data (Schuler *et al*, 2001).

Interestingly, $GABA_{B(1)}^{-/-}$ mice behave similarly to their wild-type controls in the tail suspension test. The tail suspension test is another well-characterized test for assessing depression- and antidepressant-like activity (Cryan *et al*, 2001, 2002, 2003b; Porsolt, 2000). Although this test is similar to the FST in the constructs that it purports to assess (immobility) and for its ability to detect a broad spectrum of antidepressants (Steru *et al*, 1985), it is becoming clear that both tests are probably different from each other in terms of the biological substrates that underlie their observed behaviors (Bai *et al*, 2001; Cryan and Mombereau, 2004; Renard *et al*, 2003). Accordingly, it is believed that using both paradigms can give complementary and/or converging information on activities of novel potential antidepressants or molecular pathways including those altered in genetically modified animals (Bai *et al*, 2001; Conti *et al*, 2002; Cryan *et al*, 2003b; Porsolt, 2000). The current data are among the first to show differential effects of a genetic modification in the FST and the tail suspension test, and confirm the assertion of a differential neurochemical underpinning to each test.

In order to confirm the antidepressant-like phenotype of the $GABA_{B(1)}^{-/-}$ mice pharmacologically, we assessed the effects of the $GABA_B$ receptor antagonist CGP56433A in the FST. Our data demonstrate that this $GABA_B$ receptor antagonist when administered acutely also decreases immobility in the FST without having any significant change in locomotor activity (Figure 9). Chronic administration of CGP56433A also produced an antidepressant-like effect similar to that of the antidepressant desipramine (Figure 10). Although accumulating evidence implicates GABAergic dysfunction in depression (Brambilla *et al*, 2003; Krystal *et al*, 2002), evidence for a specific role for $GABA_B$ receptors in depression and in the mechanism of action of antidepressants is limited and controversial, with rival hypotheses being purported that both positive and negative modulation of this receptor may be a useful antidepressant therapy (Lloyd *et al*, 1987; Nakagawa *et al*, 1999). Of late, more emphasis has been placed on $GABA_B$ receptor antagonism as a potential therapeutic strategy for depression (Bowery *et al*, 2002). In support of this, antidepressant-like effects were reported after chronic treatment with the $GABA_B$ receptor antagonist CGP51176 in the chronic mild stress model of depression in rats and in the rat FST (Bittiger *et al*, 1996). Further, using the learned helplessness model, it has been shown that the $GABA_B$ receptor antagonist CGP36742 had an antidepressant-like response (Nakagawa *et al*, 1999), whereas baclofen increased susceptibility to helplessness and attenuated the effects of antidepressants (Nakagawa *et al*, 1996a, b). Furthermore, baclofen also reduced the efficacy of antidepressants in the FST (Nakagawa *et al*, 1996c). Of note, $GABA_B$ receptor antagonists (including CGP56433A) increase BDNF expression in the hippocampus and cortex (Heese *et al*, 2000), which may contribute to their antidepressant-like effects (Conti *et al*, 2002; Shirayama *et al*, 2002). Taken together, our current data support the contention that antagonism of $GABA_B$ receptors may be a suitable target for the development of antidepressant agents.

Superficially at least, it may seem counterintuitive that modulation of a given receptor may induce a differential effect on anxiety- and depression-like behaviors, given the extensive comorbidity of such disorders clinically (Moller, 2002). However, $GABA_B$ receptors are localized both pre- and postsynaptically, and the elucidation of the relative contribution of these individual receptor populations to behavioral phenotypes is currently not possible. Interestingly, mice lacking the 65 kDa isoform of glutamic acid decarboxylase (GAD65), which plays an essential role in GABA synthesis, have a similar phenotype to $GABA_{B(1)}^{-/-}$ mice (increased anxiety and decreased depression-related behavior; Stork *et al*, 2000, 2003). $GAD65^{-/-}$ mice have a deficit in the temporal increase in GABA synthesis, which occurs postnatally in wild-type animals. It is tempting to speculate that the phenotype of these mice may be in part related to insufficient agonist occupancy at $GABA_B$ receptors especially during critical postnatal periods. Also of note is the fact that such a behavioral pattern is also observed in mice lacking the $5\text{-}HT_{1A}$ receptor (Ramboz *et al*, 1998) and in mice overexpressing CRF (van Gaalen *et al*, 2002). $GABA_B$ receptors are densely localized on, and intricately interact with, serotonergic neurons in the dorsal raphe nucleus (DRN) (Abellan *et al*, 2000a, b; Burman *et al*, 2003; Serrats *et al*, 2003; Tao *et al*, 1996). Given that serotonin can modulate anxiety and depression in opposite manners, with high serotonergic activity being associated with anxiety and low activity with depression (Cryan and Leonard, 2000; Graeff *et al*, 1996), it is plausible that differential interaction of $GABA_B$ receptors on 5-HT neuronal firing at the level of the DRN may be in part responsible for the behavioral effects subsequent to genetic and pharmacological manipulations of $GABA_B$. However, future studies are needed to understand the functional interactions of $GABA_B$ receptors with 5-HT and with other neurotransmitter systems and how these may contribute to the manifestation of differential anxiolytic- and antidepressant-like effects of $GABA_B$ receptor positive allosteric modulators and antagonists, respectively.

In conclusion, the current results demonstrate that $GABA_B$ receptors are important regulators of emotional behavior. However, we acknowledge both the inherent difficulties and the caution needed in the interpretation of behavioral analysis of genetically modified mice such as the $GABA_{B(1)}^{-/-}$ mice, which have overt behavioral disturbances, in more defined tests relevant to psychopathology. Nonetheless, the current data show that even such mice can still be utilized to give important indicators of the role of a given protein, in this case the $GABA_B$ receptor, in a molecular pathway relevant for the manifestation of anxiety or depression. These assertions can then be confirmed more

parametrically using appropriate pharmacological activators and antagonists as we have done using novel $GABA_B$ receptor positive modulators or antagonists.

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Research Papers

Validation of open : closed arm entries in an elevated plus-maze as a measure of anxiety in the rat

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A novel test for the selective identification of anxiolytic and anxiogenic drug effects in the rat is described, using an elevated +-maze consisting of two open arms and two enclosed arms. The use of this test for detecting such drug effects was validated behaviourally, physiologically, and pharmacologically. Rats made significantly fewer entries into the open arms than into the closed arms, and spent significantly less time in open arms. Confinement to the open arms was associated with the observation of significantly more anxiety-related behaviours, and of significantly greater plasma corticosterone concentrations, than confinement to the closed arms. Neither novelty nor illumination was a significant contributor to the behaviour of the rats on the +-maze. A significant increase in the percentage of time spent on the open arms and the number of entries into the open arms was observed only within clinically effective anxiolytics (chlordiazepoxide, diazepam and, less effectively, phenobarbitone). Compounds that cause anxiety in man significantly reduced the percentage of entries into, and time spent on, the open arms (yohimbine, pentylenetetrazole, caffeine, amphetamine). Neither antidepressants nor major tranquilisers had a specific effect. Exposure to a holeboard immediately before placement on the +-maze showed that behaviour on the maze was not clearly correlated either with exploratory head-dipping or spontaneous locomotor activity.

Introduction

In recent years, the growing realisation that several effects of the benzodiazepines are undesirable when given clinically (in particular their sedative, amnesic and dependence-inducing characteristics), has spurred the development of several new

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classes of potential anxiolytic compounds. The need for novel tests to detect anxiolytic activity has thus become pressing. Current widely-used tests of anxiety frequently involve the utilisation of noxious stimuli, such as electric shock, to condition fear in the animal, and of procedures such as food or water deprivation. The latter procedures may alter the animal's response to drugs and often necessitate additional testing to control for effects on food and water consumption; moreover, these procedures are invalid to test any compound that interferes with appetitive behaviour per se. The purpose of the present investigation was to describe a simple method of measuring anxiety in the rat that requires one five-minute test only, and that relies solely on spontaneous activity.

A further problem with current animal tests of anxiety is that they are rarely validated, other than pharmacologically, to ensure that the behaviour measured reflects anxiety in a specific fashion. A major reason for this is that such tests are extremely difficult to validate, both behaviourally and physiologically, since no one behavioural or physiological measure reliably reflects anxiety alone. However, without any attempt at such a validation, it is possible to have tests that are reliable in that they identify benzodiazepine-like compounds; but it is not clear that they can distinguish, for example, between the sedative and anxiolytic effects of these compounds, i.e. they may not be valid animal models of anxiety. The current investigation, therefore, considers the behavioural and physiological validation of the test described, as well as its pharmacological validation.

The test is based on a procedure used by Montgomery in 1958, in which he showed that exposure to an elevated (open) maze alley evoked an approach-avoidance conflict that was considerably stronger than that evoked by exposure to an enclosed maze alley. Rats that were placed in a living cage and allowed access to an enclosed alley explored considerably more than those allowed access to an open alley, and in the latter condition there were more retreats to the end of the cage. Using a Y-maze with a varying ratio of open : enclosed arms, Montgomery showed that animals clearly preferred the enclosed arms in all cases. The interpretation of this behaviour proposed by Montgomery was that exposure to novel stimuli (in the form of maze alleys) can evoke both exploratory drive and fear drive, thus generating approach-avoidance conflict behaviour, and that elevated maze alleys evoke a greater strength of fear, and therefore more avoidance behaviour, than enclosed alleys.

The present investigation explores the possibility that these discoveries can be incorporated into a method for the identification of drug effects on anxiety. Handley and Mithani (1984a) described a procedure based on that of Montgomery (1958) in which they used a +-maze with two open and two enclosed arms, and considered as a measure of anxiety the extent to which drugs selectively affect the ratio of open to enclosed arms. For example, α_2-agonists selectively increased exploration of the two open arms and thus were considered as anxiolytics; whilst α_2-antagonists selectively decreased exploration in the open arms and thus were considered to be anxiogenic. Our studies are also carried out using a +-maze.

Our first task was to ensure that the behaviour measured in this situation is a specific reflection of anxiety. That drugs can selectively affect entries into the open

arms would suggest that the behaviour being measured is not simply a change in exploratory tendencies; however, we wished to show that anxiety is greater when rats are exposed to the open arms. To do this we carried out the experiments described in the behavioural and physiological validation sections below.

If a behaviour specifically reflects anxiety, it should be possible to manipulate it specifically with drugs that affect anxiety and not with drugs affecting other behavioural systems. In the current procedure, we would expect an anxiolytic drug to selectively increase exploration of the open arms, without an equivalent increase in exploration of the enclosed arms, and an anxiogenic agent to selectively decrease exploration of the open arms without an equivalent decrease in exploration of the enclosed arms. Typical members of two classes of clinically effective anxiolytic compounds were therefore tested: chlordiazepoxide and diazepam, two benzodiazepines, and phenobarbitone, a barbiturate. These compounds were investigated after both acute and chronic (5 days) treatment, since clinically they are effective chronically, and in some animal tests their anxiolytic efficacy is increased after chronic pretreatment (File and Hyde, 1979; Margules and Stein, 1968). In addition, the effects of two compounds that have anxiogenic activity in man were investigated: pentylenetetrazole, a convulsant compound believed to act at the picrotoxinin site on the GABA-benzodiazepine receptor complex in the CNS (see Olsen, 1982) that at subconvulsant doses induces anxiety both in man and animals (Rodin and Calhoun, 1970; File and Lister, 1984; Prado de Carvalho et al., 1983), and yohimbine, an α_2-adrenoceptor antagonist that causes anxiety in man (Charney et al., 1983) and animals (Pellow et al., 1985; Handley and Mithani, 1984b). Two stimulant compounds, amphetamine and caffeine, that also have anxiogenic activity in animals (File and Hyde, 1979) and in man (Charney et al., 1984; Uhde et al., 1984; Turner and Richens, 1982) were also tested.

It is also important to know to what extent the test is likely to identify false positives. For this reason, typical representatives of other classes of psychoactive compounds were also investigated: haloperidol, a major tranquiliser that has sedative properties, and imipramine and mianserin, the former a tricyclic antidepressant and the latter an atypical antidepressant.

The test took place for 5 minutes; this time was chosen because Montgomery (1958) showed that the avoidance behaviour was particularly marked over this time period but began to decrease towards the end of a 10-minute period. Immediately before being tested in the +-maze for drug effects, each animal was placed in a holeboard for a 5-min test. This method allows the separation of directed exploration (head-dipping) from locomotor activity and rearing (File and Wardill, 1975). A comparison of the two tests would enable the determination of to what degree behaviour on the +-maze reflects changes in the exploration or locomotor activity of the animals. Pilot studies had also shown that animals placed in a novel environment before exposure to the +-maze tended to increase the overall activity in the +-maze, and to increase the likelihood that the open arms would be explored.

Materials and Methods

Animals

Animals were male hooded Lister rats (Olac Ltd., Bicester, U.K.) weighing between 250 and 400 g, except as noted in the procedure section. They were housed in groups of 5–7 in a room with an 11 h light : 13 h dark cycle, with food and water freely available.

Apparatus

The +-maze consisted of two open arms, 50 × 10 cm, and two enclosed arms, 50 × 10 × 40 cm, with an open roof, arranged such that the two open arms were opposite to each other. The maze was elevated to a height of 50 cm. The measures indicated in the procedure section were taken by two observers, sitting in the same room as the maze.

The holeboard was a wooden box, 60 × 60 × 35 cm, with four holes of 3.8 cm diameter equally spaced in the floor. Infrared photocells in the sides of the box, 4.5 and 11 cm from the floor, provided measures of locomotor activity and of the number of rears, respectively. Photocells below the surface of the holes provided measures of the number of head-dips and the time spent head-dipping. All measures were entered directly into a Control Universal microcomputer.

Drugs

Chlordiazepoxide (CDP, Roche Products), pentylenetetrazole (PTZ, Sigma), yohimbine (Sigma), D-amphetamine (Sigma), mianserin (Beecham) and imipramine (Sigma) were dissolved in distilled water. Diazepam (Roche Products), sodium phenobarbitone (BDH chemicals Ltd.), caffeine (Sigma) and haloperidol (Searle) were suspended in distilled water with a drop of Tween 20. All drugs were injected intraperitoneally 30 min before testing, except for PTZ which was injected 5 min before, in concentrations to give an injection volume of 2 ml/kg.

Procedure

Behavioural validation

Different strains of rat

To see whether strain differences are important in this test, two groups of animals (n = 6–8) were tested from the following strains of rat: hooded Lister (Olac, Ltd., Bicester, UK) and Wistar rats (Charles River, France). The test procedure was as follows: rats were placed individually in the centre of the maze, and the following measures scored by two observers: number of entries into (a) open and (b) closed arms; the time spent in (a) open and (b) closed arms. An arm entry was defined (as in all following experiments) as the entry of all four feet into one arm. Student's *t*-tests were performed to determine whether there was a significant difference between the number of entries into the closed and into the open arms, and between the time spent in the closed and in the open arms, for each individual strain.

First choice of alley type

Two groups of animals (n = 8) were tested; in the first, animals were placed on the centre of the maze facing an open arm, and in the second, facing an enclosed arm. In both cases the first arm that the animal entered was noted. It was expected that if open arms were more aversive, animals would be less likely to make them their first choice. Data were analysed using a Fisher exact probability test.

Effects of repeated testing

On three consecutive days, 6 rats were placed individually in the centre of the maze, and the following measures scored: number of entries into open arms; time spent on open arms; number of entries into closed arms; time spent in enclosed arms. This experiment would enable us to see whether behaviour on the open arms increased with increasing familiarity, and would also permit the calculation of the test-retest reliability of the procedure.

Data were analysed by one-way analyses of variance, with the percentage of open arm entries, the time spent in the open arms as a percentage of the total, or the total number of alley entries as the measures and days as the factor.

Effects of light level in enclosed arms

Two groups of rats (n = 6) were placed individually in the centre of the maze and the measures described above were scored for 5 min under two conditions. In the first, the condition that was used for all other experiments, an angle-poise lamp was placed to one side of the maze such that the light level on the open arms was greater than that in the enclosed arms due to the shadows cast by the latter. In the second, the light level in the enclosed arms was increased by placing a desk lamp in the centre of the maze such that the light level in the two arms was approximately equivalent. We hoped thereby to discover the extent to which the animal's preference for enclosed arms is due to a lower light level under normal lighting conditions.

Data were analysed by ANOVAs as described above, with light level as the factor.

Measurement of anxiety-related behaviours

Six rats were individually confined either to the two open arms or the two enclosed arms of the maze, and the following behaviours scored: (1) displacement activity (i.e. grooming, gnawing, chewing of non-edible objects), (2) freezing i.e. absolute stillness, with no movement even of the whiskers, (3) immobility i.e. stillness but with some movement of the whiskers and (4) defaecation.

Data were analysed by Student's *t*-tests.

Physiological validation

Increased plasma corticosterone concentrations have been observed in animals after exposure to several anxiogenic/stressful procedures (Hennessy and Levine, 1979; File, 1980). Blood was taken by direct cardiac puncture from singly-housed

rats that had been briefly anaesthetised with ether and that were allocated to each of the following conditions (n = 6 per group): (1) rats were taken from their home cages and immediately sampled; (2) rats were confined to the two enclosed arms of the maze for 20 min and then sampled; (3) rats were confined to the two open arms of the maze for 20 min and then sampled. The 20-min time-point was selected because this is the time necessary for corticosterone levels to peak after a change in test conditions (see Hennessy and Levine, 1979; File, 1980). All samples were collected between 08.00 and 10.00 h. The blood was collected in heparinised plastic tubes, centrifuged at 3000–3500 rpm and the plasma stored at −20°C for later assay. Plasma concentrations of corticosterone were determined using a fluorimetric assay (Zencker and Bernstein, 1958).

Data were analysed by analysis of variance, with test condition as the factor. Posthoc comparisons between individual groups were made using Dunnett's *t*-tests or Duncan's Multiple Range tests, as appropriate.

Pharmacological validation

The following procedure was used throughout. Each rat received an injection (i.p.) and was then returned to his home cage. 30 min afterwards (5 min for PTZ) the animal was placed in the centre of the holeboard and given a 5-min trial. The rat was then immediately placed in the centre of the maze, facing one of the open arms. Two observers in the same room scored the following behaviours for 5 min: (1) the first choice of arm entry (i.e. open or closed), (2) the number of entries into each type of arm, (3) the total time spent in each type of arm. Both the holeboard and the maze were thoroughly wiped clean after each trial.

Animals were randomly allocated to the following groups (n = 8). (1) Acute studies: (a) vehicle control (n = 16): CDP (5 or 7.5 mg/kg); phenobarbitone (25 or 35 mg/kg); yohimbine (1.25 or 2.5 mg/kg); amphetamine (1 or 2 mg/kg); imipramine (5 or 15 mg/kg); mianserin (10 or 20 mg/kg); (b) vehicle control: diazepam (1 or 2 mg/kg); (c) vehicle control (n = 17): caffeine (15 or 30 mg/kg); haloperidol (0.1 or 0.25 mg/kg). (2) Chronic studies: animals received an injection of one of the following for 5 days before testing, and on the test day: (a) vehicle control, CDP (5 or 7.5 mg/kg), phenobarbitone (25 or 35 mg/kg); (b) vehicle control, diazepam (1 or 2 mg/kg).

First arm entry was analyzed using Fisher-Yates exact probability tests.

ANOVAs were performed on the percentage of open arm entries or time spent in the open arms, as described in Behavioural validation above, with drug treatment as the factor. Where a drug decreased both open and closed arm entries or the time spent in both types of arm, analysis of covariance was performed with open arm entries (or time) as the dependent variable, and closed arm entries (or time) as the covariate.

ANOVAs were performed on the total number of arm entries, with drug treatment as the factor.

Holeboard data were analysed by ANOVA with drug treatment as the factor.

Results

Behavioural validation

Different strains of rat

Results from the two different strains of rat are shown in Table I. In both strains, the number of open arm entries and the time spent in the open arms was significantly lower than the same measures taken in the closed arms (significance values in Table I).

Choice of alley type

In rats placed on the maze facing an open alley, 5/10 chose an open alley and 5/10 a closed alley as their first entry. In those placed facing a closed alley, 2/10 chose an open alley and 8/10 a closed alley. There were no significant differences between the two groups.

Effects of repeated testing

ANOVA showed that there was no significant difference in the percentage of open arm behaviour over the 3 days of testing, either on the number of entries ($F_{(2,15)} = 0.13$) or the time ($F_{(2,15)} = 0.07$), see Table II. Similarly, there were no significant changes in total entries ($F_{(2,15)} = 0.14$); see Table II. Table II also shows the correlation coefficients for the test-retest correlations of results obtained on days 2 and 3 with those obtained on day 1. On both days 2 and 3 the correlation coefficient was high, although this was less markedly true by day 3.

TABLE I

A COMPARISON OF THE BEHAVIOUR OF TWO STRAINS OF RAT ON A 5-MIN TEST ON THE +-MAZE

Scores are mean (±S.E.M.) number of entries and time (s) spent in open or in closed arms; total number of entries; the percentage of entries or of time spent in the open arms. For strains, see methods.

	Olac hooded	Wistar
Closed		
Number	8.5 ± 1.71	7.6± 1.20
Time	181.3 ±18.67	190.2±14.78
Open		
Number	2.0 ± 0.63 *	2.2± 0.86 *
Time	28.6 ± 9.30 **	24.0±13.60 **
Total		
Number	10.4 ± 1.84	9.8± 1.59
Open / total		
Number	17.2 ± 5.46	20.5± 6.93
Time	14.35± 4.30	11.5± 6.47

* $P < 0.01$, Students *t*-test, compared with closed arms.
** $P < 0.001$, Students *t*-test, compared with closed arms.

TABLE II

THE EFFECTS OF REPEATED TESTING OVER 3 DAYS IN RATS PLACED ON AN ELEVATED +-MAZE FOR 5 MIN DAILY

Scores are mean (±S.E.M.) number of entries or time (s) spent in closed arms, open arms, or in all arms, and the percentage of entries or of time spent in the open arms. The correlation coefficients for the total arm entries, and for the percentage of entries or of time spent in the open arms, are given for days 2 and 3 with day 1.

	Day 1	Day 2	Day 3
Closed			
Number	6.0± 0.85	6.8± 1.47	6.3± 1.28
Time	193.8±26.51	207.0±36.35	179.5±27.82
Open			
Number	4.0± 1.69	5.3± 2.23	4.6± 1.52
Time	49.1±21.53	59.2±26.65	57.3±18.98
Total			
Number	10.0± 2.48	12.2± 3.61	11.0± 2.50
		$r = 0.98$	$r = 0.85$
Open/total			
Number	28.1± 8.89	29.9± 9.09	34.9± 8.46
		$r = 0.99$	$r = 0.97$
Time	20.8± 6.21	24.2± 9.96	25.7± 7.64
		$r = 0.79$	$r = 0.70$

Effects of light level

ANOVA showed there was no significant change in open arm behaviour as compared to closed arm behaviour as a function of equalising the light level in the two types of arm, either on number of entries or time spent on open arms. Similarly, there were no significant changes in total arm entries, see Table III.

TABLE III

MEAN (±S.E.M.) NUMBER OF ENTRIES OR TIME (s) SPENT IN CLOSED ARMS, OPEN ARMS OR ALL ARMS, AND THE OPEN:TOTAL ENTRY RATIO, FOR RATS GIVEN A 5 MIN TEST IN A +-MAZE, WITH BRIGHT LIGHT ON OPEN ARMS ONLY, OR BRIGHT LIGHT IN ALL FOUR ARMS

	Open	All arms
Open		
Number	5.2± 0.83	5.8± 1.28
Time	66.7±12.13	62.0±11.42
Closed		
Number	8.8± 1.52	10.3± 1.02
Time	123.7±10.22	117.5± 7.33
Total		
Number	14.0± 2.16	16.2± 2.18
Open/total		
Number	37.6± 3.36	33.2± 4.37
Time	33.9± 4.26	33.6± 5.68

TABLE IV

MEAN (±S.E.M.) NUMBER OF ENTRIES FOR TIME (s) SPENT FREEZING OR IMMOBILE, TIME SPENT GROOMING AND NUMBER OF BOLUSES, IN RATS GIVEN A 5 MIN TEST EITHER ON TWO OPEN ARMS OR TWO CLOSED ARMS

	Open arms	Closed arms
Entries	3.50±0.42 **	7.00±0.73
Immobility	78.16±6.20 ***	30.33±5.74
Freezing	71.33±7.02 **	30.83±8.34
Grooming	1.16±0.82	0.5 ±0.5
Boluses	3.50±0.93 *	1.16±0.65

* $P < 0.05$,
** $P < 0.01$,
*** $P < 0.001$, significantly different from groups in enclosed arms, Student's t-test.

Measurement of anxiety-related behaviours

Table IV shows that there was a significant decrease in the number of entries ($P < 0.01$), and a significant increase in the time spent motionless ($P < 0.001$) and time spent freezing ($P < 0.01$), and in the number of boluses produced ($P < 0.05$) by rats exposed to open arms as compared with closed arms. Little displacement activity was observed in either case, see Table IV.

Physiological validation

ANOVA showed a significant effect on plasma corticosterone concentrations as a function of test condition ($F_{(2,15)} = 26.53$, $P < 0.0001$); posthoc analysis showed that rats confined to both the open and closed arms had significantly elevated corticosterone levels compared with home-cage controls ($P < 0.01$); in addition, rats confined to the open arms had significantly elevated corticosterone concentrations compared to those confined to closed arms ($P < 0.01$). The mean (±S.E.M.) plasma corticosterone concentrations (μg/100 ml) were: controls, 7.6 ± 1.83; closed arms, 24.4 ± 3.15; open arms, 51.6 ± 6.51.

Pharmacological validation

+-Maze

Figs. 1–5 show the open arm entries and the amount of time spent on the open arms expressed as a percentage of the total entries or time. Tables V and VI show the total number of entries into the arms, and the number of rats who chose to enter a closed arm first.

CDP, given acutely (5–7.5 mg/kg), had an effect to reduce the total number of arm entries that just missed significance on the ANOVA ($F_{(2,29)} = 2.89$, $P = 0.07$). When given chronically, CDP did not significantly affect this measure ($F_{(2,21)} = 0.14$). Acute treatment with CDP significantly elevated the percentage of open arm entries ($F_{(2,29)} = 11.97$, $P < 0.0005$) and the time spent in the open arms ($F_{(2,29)} = 4.47$

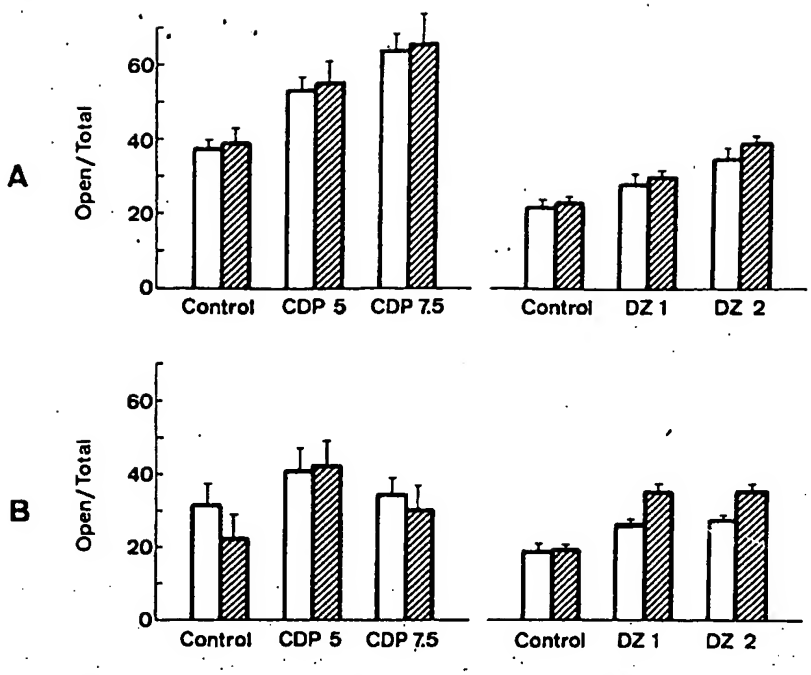

Fig. 1. Mean (±S.E.M.) percentage of open arm entries (white bars) or of time (s) spent in the open arms (hatched bars) in rats placed at the centre of a plus-maze and given a 5-min test, immediately after a 5-min test in the holeboard. 30 min previously rats were injected with vehicle control, chlordiazepoxide (5 or 7.5 mg/kg), diazepam (1 or 2 mg/kg), (A) after a single acute treatment or (B) after 5 days pretreatment.

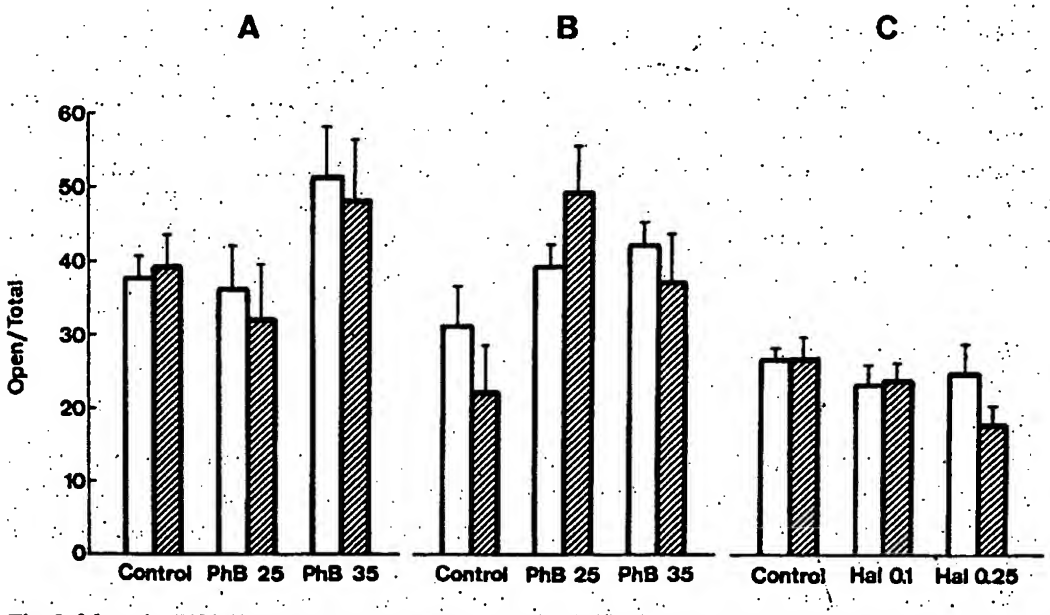

Fig. 2. Mean (±S.E.M.) percentage of open arm entries (white bars) or of time (s) spent in the open arms (hatched bars) in rats placed at the centre of a plus-maze and given a 5-min test, immediately after a 5-min test in the holeboard. 30 min previously rats were injected with vehicle control, phenobarbitone (25 or 35 mg/kg) (A) after a single acute treatment, (B) after 5 days pretreatment, or (C) with haloperidol (0.1–0.25 mg/kg).

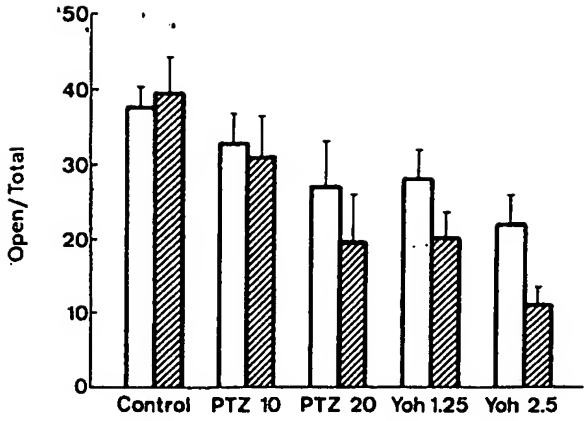

Fig. 3. Mean (±S.E.M.) percentage of open arm entries (white bars) or of time (s) spent in the open arms (hatched bars) in rats placed at the centre of a plus-maze and given a 5-min test, immediately after a 5-min test in the holeboard. Rats had previously been injected with vehicle control, pentylenetetrazole (10 or 20 mg/kg) or yohimbine (1.25-2.5 mg/kg).

$P < 0.05$). Chronically, CDP no longer caused a significant elevation in the percentage of open arm entries ($F_{(2,23)} = 1.12$); the elevation in the percentage of time spent in these arms just missed significance ($F_{(2,23)} = 2.77$, $P = 0.08$).

Diazepam (1–2 mg/kg), given acutely, reduced the number of arm entries ($F_{(2,21)} = 14.23$, $P < 0.0001$); when given chronically there was no significant reduction ($F_{(2,21)} = 2.49$). After acute treatment with diazepam, the elevation in the percentage of open arm entries just missed significance ($F_{(2,27)} = 2.51$; $P = 0.09$); however, there was a significant elevation in the the time spent in the open arms ($F_{(2,27)} = 3.64$, $P < 0.05$). Chronically, diazepam also elevated both these measures ($F_{(2,21)} = 7.55$, $P < 0.005$ and $F_{(2,21)} = 19.97$, $P < 0.0001$, respectively).

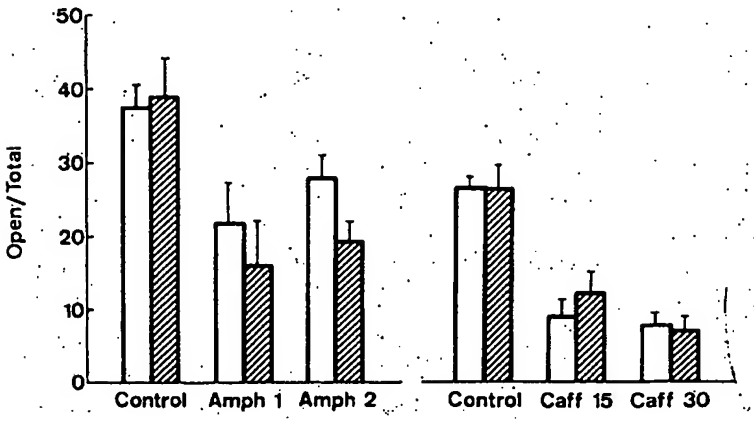

Fig. 4. Mean (±S.E.M.) percentage of open arm entries (white bars) or of time (s) spent in the open arms (hatched bars) in rats placed at the centre of a plus-maze and given a 5-min test, immediately after a 5-min test in the holeboard. Rats had been injected 30 min previously with vehicle control, amphetamine (1–2 mg/kg) or caffeine (15–30 mg/kg).

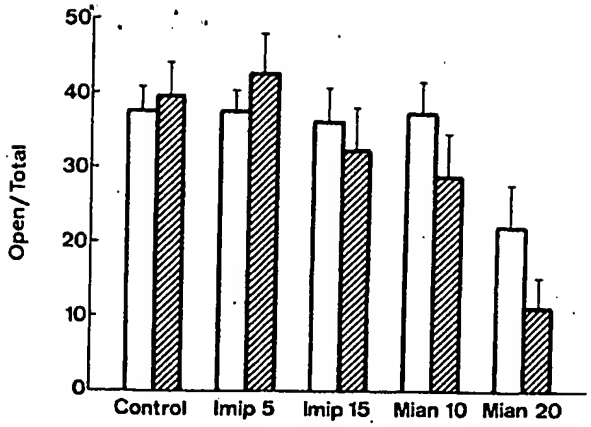

Fig. 5. Mean (±S.E.M.) percentage of open arm entries (white bars) or of time (s) spent in the open arms (hatched bars) in rats placed at the centre of a plus-maze and given a 5-min test, immediately after a 5-min test in the holeboard. 30 min previously rats were injected with vehicle control, imipramine (5 or 15 mg/kg) or mianserin (10 or 20 mg/kg).

Phenobarbitone, given acutely or chronically (25–35 mg/kg), reduced the total number of arm entries ($F_{(2,29)} = 3.64$, $P < 0.05$; and $F_{(2,19)} = 4.67$, $P < 0.05$, respectively). Acute treatment with phenobarbitone had no significant effect either on the percentage of entries into open arms to the time spent in open arms ($F_{(2,29)} = 2.17$

TABLE V

MEAN (±S.E.M.) TOTAL NUMBER OF ARM ENTRIES AND FIRST CHOICE OF TYPE OF ARM ENTRY, IN RATS GIVEN A 5-MIN TEST AFTER TREATMENT WITH THE ANXIOLYTIC COMPOUNDS CHLORDIAZEPOXIDE, DIAZEPAM AND PHENOBARBITONE, GIVEN ACUTELY OR CHRONICALLY (5 DAYS PRETREATMENT)

Drug (mg/kg)		Total entries	Choice of closed arm
(A) ACUTE			
Control		16.2±1.13	8/16
Chlordiazepoxide	5.0	12.5±1.42	5/8
	7.5	12.1±1.60	5/8
Phenobarbitone	25.0	13.4±2.02	3/8
	35.0	10.2±1.61 *	4/8
Control		13.4±0.62	5/8
Diazepam	1.0	12.6±0.65	4/8
	2.0	8.8±0.63 ****	3/8
(B) CHRONIC			
Control		18.6±2.16	3/8
Chlordiazepoxide	5.0	17.1±2.35	4/8
	7.5	18.5±1.52	7/8
Phenobarbitone	25.0	13.4±2.07	3/8
	35.0	10.2±1.61 *	4/8
Control		13.4±0.53	5/8
Diazepam	1.0	16.5±0.75	3/8
	2.0	19.4±0.67	3/8

* $P < 0.05$.
**** $P < 0.0001$.

TABLE VI

MEAN (±S.E.M.) TOTAL NUMBER OF ARM ENTRIES AND FIRST CHOICE OF ARM ENTRY, IN RATS GIVEN A 5-MIN TEST AFTER TREATMENT WITH THE ANXIOGENIC COMPOUNDS PENTYLENETETRAZOLE, YOHIMBINE, AMPHETAMINE AND CAFFEINE; THE ANTIDEPRESSANTS IMIPRAMINE AND MIANSERIN, AND THE MAJOR TRANQUILISER HALOPERIDOL

Control		16.2±1.13	8/16
Pentylenetetrazole	10.0	12.9±1.23	7/8
	20.0	8.6±1.50 ***	8/8
Yohimbine	1.25	12.8±0.96	7/8
	2.5	10.9±0.72 **	6/8
Amphetamine	1.0	20.1±2.57	5/8
	2.0	16.5±2.32	5/8
Imipramine	5.0	16.9±1.58	4/8
	15.0	11.1±1.41 *	2/8
Mianserin	10.0	13.5±2.43	4/8
	20.0	9.9±1.74 *	6/8
Control		13.5±0.57	13/17
Caffeine	15.0	14.8±1.20	8/9
	3.0	16.7±0.95 *	9/9
Haloperidol	0.1	9.5±0.66	6/9
	0.25	5.8±0.68 ****	5/9

* $P < 0.05$.
** $P < 0.005$.
*** $P < 0.0005$.
**** $P < 0.0001$.

and 0.93, respectively), but after chronic treatment there was a significant elevation of the percentage of time spent in the open arms ($F_{(2,21)} = 5.22$, $P < 0.05$) but not the percentage of arm entries ($F_{(2,21)} = 2.31$).

Yohimbine (1.25–2.5 mg/kg) significantly reduced the total number of arm entries ($F_{(2,29)} = 5.56$, $P < 0.01$). Both the percentage of open arm entries ($F_{(2,29)} = 4.21$, $P < 0.05$) and the percentage of time spent in the open arms ($F_{(2,29)} = 9.92$, $P < 0.0005$) were also reduced. Although this drug reduced the number of entries into both types of arm, analysis of covariance showed that there was a decrease in the entries into the open arms independently of the decrease in closed arm entries ($F_{(2,28)} = 7.18$, $P < 0.005$).

PTZ (10–20 mg/kg) significantly reduced the total number of arm entries ($F_{(2,29)} = 8.06$, $P < 0.005$). There was no significant reduction in the percentage of open arm entries ($F_{(2,29)} = 1.47$) but a reduction in the percentage of time spent in the open arms was obtained that was marginally significant ($F_{(2,29)} = 2.95$, $P < 0.07$). PTZ reduced arm entries into both types of arm, and analysis of covariance showed that the decrease in open arm entries was not independent of the decrease in closed arm entries ($F_{(2,28)} = 1.32$).

Amphetamine (1–2 mg/kg) had no significant effect on the total arm entries ($F_{(2,29)} = 1.35$). However, this compound caused a significant reduction both in the percentage of open arm entries ($F_{(2,29)} = 4.01$, $P < 0.05$) and of time spent in the

open arms ($F_{(2,29)} = 6.46$, $P < 0.005$). Caffeine (15–30 mg/kg) significantly elevated the total number of arm entries ($F_{(2.32)} = 3.58$, $P < 0.05$); this compound caused a significant decrease both in the percentage of open arm entries ($F_{(2,32)} = 30.33$, $P < 0.0001$) and the percentage of time spent in the open arms ($F_{(2,32)} = 12.85$, $P < 0.0001$).

Haloperidol (0.1–0.25 mg/kg) significantly reduced the total number of arm entries ($F_{(2,32)} = 37.41$, $P < 0.0001$); but had no significant effect on the percentage of open arm entries or of time spent in the open arms ($F_{(2,32)} = 0.55$ and 2.51, respectively). Analysis of covariance showed that the decrease in open arm entries was correlated with the decrease in closed arm entries ($F_{(2,31)} = 1.95$).

Mianserin (10–20 mg/kg) significantly decreased the total number of arm entries made ($F_{(2,29)} = 3.47$, $P < 0.05$). Surprisingly, this compound also significantly reduced the percentage of open arm entries ($F_{(2,29)} = 3.43$, $P < 0.05$) and of time spent on open arms ($F_{(2,29)} = 6.73$, $P < 0.005$); however, analysis of covariance showed that the decrease in open arm entries produced by this compound was not independent of the decrease in closed arm entries ($F_{(2,28)} = 2.45$).

Imipramine (5–15 mg/kg) significantly reduced the total number of arm entries made by rats ($F_{(2,29)} = 4.03$, $P < 0.05$), but there was no significant effect on the percentage of open arm entries or of time spent in open arms ($F_{(2,29)} = 0.05$ and 0.69,

TABLE VII

MEAN (±S.E.M.) NUMBER OF HEAD-DIPS, TIME (s) SPENT HEAD-DIPPING, LOCOMOTOR ACTIVITY SCORE AND NUMBER OF REARS, FOR RATS GIVEN A 5 MIN TEST AFTER TREATMENT WITH THE ANXIOLYTIC COMPOUNDS CHLORDIAZEPOXIDE, PHENOBARBITONE AND DIAZEPAM

Drug (mg/kg)	No.	Time	Motor	Rears
(A) ACUTE				
Control	11.0±1.30	12.7±4.09	270.4±23.70	36.9±2.87
CDP 5	7.5±2.67	8.7±3.07	214.0±45.42	24.4±5.88
7.5	3.7±1.25 *	2.8±0.92	144.1±31.58 *	14.5±4.12 **
PhB 25	10.1±2.50	6.7±1.54	201.4±35.66	24.5±4.97
35	8.7±2.16	8.4±1.96	122.0±28.17 **	20.7±3.97 *
Control	15.4±1.29	14.5±1.78	330.6±24.87	30.0±2.85
DZ 1	12.2±1.33	13.0±1.05	311.9±20.89	26.0±2.22
2	5.5±1.26 ***	5.9±1.33 ****	113.6±19.62 ****	12.4±2.52 ****
(B) CHRONIC				
Control	13.1±2.13	6.7±0.82	282.9±28.97	34.6±4.94
CDP 5	11.6±2.39	6.2±1.00	327.7±30.39	35.1±2.37
7.5	12.7±2.24	10.6±2.48	259.6±33.93	32.9±5.08
PhB 25	20.9±3.06	13.0±1.41	309.6±31.57	39.6±4.74
35	14.0±1.25 *	11.3±1.99 *	231.4± 9.84	29.0±4.55
Control	13.2±1.46	13.0±1.30	261.2±17.42	32.4±3.47
DZ 1	12.7±1.03	12.6±1.74	264.5±23.69	37.0±3.67
2	13.1±1.66	12.7±2.02	245.7±18.01	35.6±4.72

* $P < 0.05$, ** $P < 0.005$, **** $P < 0.0001$.

TABLE VIII

MEAN (±S.E.M.) NUMBER OF HEAD-DIPS, TIME (s) SPENT HEAD-DIPPING, LOCOMOTOR ACTIVITY SCORE AND NUMBER OF REARS, FOR RATS GIVEN A 5 MIN TRIAL AFTER INJECTION OF THE ANXIOGENIC COMPOUNDS PENTYLENETETRAZOLE, YOHIMBINE, AMPHETAMINE AND CAFFEINE, THE ANTIDEPRESSANTS IMIPRAMINE AND MIANSERIN, AND THE MAJOR TRANQUILISER HALOPERIDOL

Drug (mg/kg)	No.	Time	Motor	Rears
Control	11.0±1.30	12.7± 4.09	270.4±23.70	36.9±2.87
PTZ 10	9.1±1.49	7.6± 1.56	305.5±26.08	31.1±3.89
PTZ 20	3.1±0.89 **	3.2± 0.99	122.2±21.61 ***	12.9±1.90 ****
YOH 1.25	3.6±0.90	3.8± 0.91	86.6±11.91	15.9±2.43
YOH 2.5	2.1±1.08 ****	2.4± 1.36	78.5± 6.72 ****	14.0±2.10 ****
Amphet 1	10.2±2.14	4.3± 1.36	348.2±44.33	45.1±4.78
Amphet 2	6.5±1.93	4.3± 2.40	363.5±19.51 *	46.1±3.35
Imip 5	13.1±1.64	9.5± 1.93	290.5±33.04	36.1±3.22
Imip 15	8.6±1.56	9.3± 1.73	145.0±18.84	19.7±2.91 **
Mian 10	6.6±1.65	3.4±81.88	165.9±30.46	23.2±3.69
Mian 20	4.5±1.06 **	2.6± 0.48	136.4±26.78 **	15.7±3.93 **
Control	10.7±0.76	11.7± 0.97	249.9±10.13	29.0±2.21
Caff 15	7.3±1.22	8.3± 1.71	334.0±25.89	34.2±4.03
Caff 30	6.1±1.07 **	8.6± 1.23	336.4±25.66 **	36.7±3.86
Halo 0.1	7.2±1.11	7.1± 1.22	189.2±12.31	21.4±3.95
Halo 0.25	3.0±0.83 ****	2.8± 0.70 ****	88.4±14.48 ****	7.4±1.47 ****

* $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$, **** $P < 0.0001$.

respectively), and analysis of covariance confirmed that the decrease in open arm entries were not independent of the decrease in closed arm entries ($F_{(2,28)} = 0.81$).

There were no significant differences between treated animals and controls on the choice of arm entry made by the animal when first placed on the maze.

Holeboard

Tables VII and VIII show the results from this test; probability levels from analysis of variance are given in the tables for each compound.

Discussion

The behavioural results suggest that rats prefer the closed arms of the maze to the open arms. Rats spend a significantly greater amount of time in the closed arms, and enter them more frequently than the open arms. Our results would agree with those of Montgomery (1958) that this preference is likely to reflect an aversion towards the open arms caused by fear or anxiety: significantly more anxiety-related behaviour was observed on the open arms (freezing, immobility, defaecation) and significantly

higher plasma corticosterone concentrations were found in rats confined to the open arms as compared with those confined to the closed arms, suggesting that exposure to the former was more stressful. Interestingly, rats exposed to the closed arms had plasma corticosterone concentrations higher than those of home cage controls, no doubt reflecting the novelty of the procedure (see Hennessy and Levine, 1979). This aversion was not consistently present as early as the first arm entry, since control rats did not show a consistent tendency to enter a closed arm first. It is therefore possible that the aversive quality of the open arms is not apparent until the rats enter it.

It appears that neither novelty nor illumination is a critical determinant of the rats' behaviour on the maze; rats did not show a lesser avoidance of the open arms after three days repeated testing; and their preference for the closed arms was not related to the level of illuminance within. It is thus likely that the major determinant of behaviour in this test is the unconditioned aversion to heights and open spaces that is consistently displayed by both laboratory and wild rats (Barnett, 1975). That the aversion to the open arms did not habituate over time was surprising, since unconditioned aversions generally habituate rapidly. However, three days may have been too short a period for habituation to take place. At any rate, this lack of rapid habituation is practically useful since it means that rats can be retested at least twice without diminishing the aversiveness of the situation. The suitability of the procedure for retesting is borne out by the high test-retest reliability that we found over the three-day test period; however, the reliability is not so good by the third day of testing, and so we suggest that, whilst this test can be repeated twice on the same animals, testing more than twice could produce unreliable results.

Our experiments with different rat strains showed that in principle both hooded and albino strains are suitable for this procedure (this is not the case, for example, with the social interaction test—File, unpublished data). However, in the course of pilot studies we found one strain of hooded rat that lacked a consistent aversion to the open arms; these were black hooded PVG rats from Banting and Kingman, U.K. Clearly, therefore, the selection of a suitable strain is important. We selected Olac rats for our other studies, and although the percentage of open arm entries showed some variation between the different control groups, this was not too marked and ranged from 20–40%.

The pharmacological studies showed that a selective increase in exploration of the open arms was observed only with drugs that are clinically effective anxiolytics: chlordiazepoxide, diazepam and, to a lesser extent, phenobarbitone. With diazepam, this effect was observed after both acute and chronic (5 days) treatment. Similarly, a selective decrease in exploration of the open arms was observed with the anxiogenic compound yohimbine. PTZ, also shown to be anxiogenic, non selectively decreased exploration of both types of arms; this is consistent with its non-specific depression of behaviour observed in the social interaction test of anxiety (File and Lister, 1984). Caffeine and amphetamine, two behavioural stimulants that have also been shown to possess anxiogenic activity in man and in other animal tests also had specific anxiogenic activity in the +-maze.

Sedative drugs, such as the major tranquiliser haloperidol, that have no well-

TABLE IX

A COMPARISON BETWEEN RESULTS OBTAINED IN THE +-MAZE (PERCENTAGE OF OPEN ARM ENTRIES OR TIME SPENT IN OPEN ARMS; TOTAL ARM ENTRIES) AND THOSE OBTAINED IN THE HOLEBOARD (NUMBER OF HEAD-DIPS OR TIME SPENT HEAD-DIPPING; MOTOR ACTIVITY)

↑, increase; ↓, decrease; –, no effect; 1, effect on time only; 2, effect on number only.

Drug	% Open	Total	Head-dipping	Motor
Chlordiazepoxide				
acute	↑	↓	↓[2]	↓
chronic	↑[1]	–	–	–
Diazepam				
acute	↑	↓	↓	↓
chronic	↑	–	–	–
Phenobarbitone				
acute	–	↓	–	↓
chronic	↑[1]	–	↑	–
Yohimbine	↓	↓	↓[2]	↓
Pentylenetetrazole	↓[1]	↓	↓[2]	↓
Amphetamine	↓	–	–	↑
Caffeine	↓	↑	↓[2]	↑
Haloperidol	–	↓	↓	↓
Imipramine	–	↓	–	↓
Mianserin	↓	↓	↓[2]	↓

established clinical anxiolytic activity and are inactive in other animal tests of anxiety, do not have a specific effect in the +-maze but non-selectively reduce overall exploration. Similarly, the antidepressants imipramine and mianserin, known to have sedative side-effects but not significant anxiolytic activity except where related to depression, reduced entries into both types of arm. It appears that the percentage of time spent in the open arms is more sensitive to drug effects than the number of entries.

Table IX compares the direction of effects of each compound tested on overall activity in the maze, percentage of open arm activity in the maze, exploration in the holeboard, and locomotor activity in the holeboard. Overall activity in the maze was correlated with exploration and locomotor activity in the holeboard, such that drugs reducing the total number of arm entries tended to reduce either or both head-dipping and motor activity in the holeboard. (The only exception to this is phenobarbitone, after chronic treatment, which decreased maze activity but increased exploratory head-dipping). It is therefore likely that the total number of arm entries is a function both of exploratory and locomotor tendencies. The percentage of open arm entries (i.e. our measure of fear/anxiety) did not correlate with holeboard behaviour. Compounds increasing the percentage of open arm entries often decreased exploration or motor activity, such as acute benzodiazepines, and compounds not affecting anxiety, such as haloperidol, antidepressants, decreased exploration and motor activity. The percentage of open arm entries/time spent on the open arms is therefore likely to specifically reflect anxiety and cannot be explained

by competing behaviours such as exploration.

In conclusion, we hope that the procedure described in this paper will provide a valid and reliable measure of anxiety that possesses several clear advantages over existing tests: (1) it is a fast and simple procedure that does not involve the use of expensive equipment; (2) it is based on spontaneous behaviour and thus does not necessitate lengthy training nor the use of noxious stimuli such as electric shock, nor manipulation of appetitive behaviours such as food and water deprivation; (3) it is able to identify the anxiolytic effects of benzodiazepine-like drugs after acute treatment and as such provides an advantage over procedures such as the social interaction test where sedation interferes with the ability to measure an anxiolytic effect; (4) It is able to identify both anxiolytic and anxiogenic drug effects under identical conditions, and thus provides an advantage over tests such as the Geller-Seifter and Vogel conflict procedures, where two quite different shock levels, and thus different behavioural baselines, are necessary to see effects in both directions.

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ORIGINAL INVESTIGATION

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Lack of effect of CCK_B receptor antagonists in ethological and conditioned animal screens for anxiolytic drugs

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Abstract The effects of the CCK_B receptor antagonists L-365,260, CI-988 and L-740,093, a new compound with improved bioavailability and CNS penetration, were assessed for anxiolytic-like effects in three rat anxiolytic screens sensitive to benzodiazepines, the elevated plus maze (EPM), conditioned suppression of drinking (CSD) and conditioned emotional response (CER) tests. In the EPM, L-740,093 (0.1–1.0 mg/kg), L-365,260 (0.00001–10.0 mg/kg), and CI-988 (0.01–1.0 mg/kg) did not increase the time spent on the open arms of the maze or the number of entries onto the open arms. In contrast, the benzodiazepine receptor partial agonist, bretazenil (0.3–10.0 mg/kg), significantly increased both the time spent on the open arms and the number of open arm entries. In the CSD and the CER tests, L-740,093 (0.1–1.0 mg/kg) L-365,260 (0.0001–0.1 mg/kg) and CI-988 (0.01–10.0 mg/kg) failed to increase suppression ratios compared to the vehicle-treated control rats, whereas, the benzodiazepine receptor partial agonist FG 8205 (10.0 mg/kg) (CSD) and bretazenil (0.3–3.0 mg/kg) (CER) both significantly increased suppression ratios compared to vehicle-treated control rats. In addition, L-365,260 (1.0–50.0 mg/kg), CI-988 (0.1–10.0 mg/kg) and diazepam (0.1–1.0 mg/kg) were assessed in a squirrel monkey conflict procedure. Although diazepam significantly increased suppressed lever pressing rates, L-365,260 and CI-988 were without effect. The present findings provide little support for the hypothesis that CCK_B receptor antagonists have anti-anxiety effects in animals.

Key words L-740,093 · L-365,260 · CI-988 · CCK_B antagonists · Anxiety · Rats · Squirrel monkeys

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Introduction

A number of studies have shown that the neuropeptide cholecystokinin (CCK) may be involved in mediating panic- or anxiety-like symptoms in humans. For example, Bradwejn et al. (1990) reported that the C-terminal tetrapeptide fragment of CCK_8, CCK_4, given intravenously, elicited panic-like symptoms in patients with panic disorder. By contrast, the intravenous injection of CCK_8-S, which does not penetrate the blood-brain barrier, induced only severe gastrointestinal symptoms (De Montigny 1989). At present, two subtypes of CCK receptors have been identified, CCK_A and CCK_B. As CCK_4 is a selective CCK_B receptor agonist (Chang and Lotti, 1986), CCK_B receptors may play a role in mediating panic symptoms and thus CCK_B receptor antagonists may have therapeutic utility as anti-panic or anti-anxiety agents.

The preclinical development of CCK_B receptor antagonists has been hampered by the lack of a well defined animal model of panic. However, selective CCK_B receptor antagonists, such as CI-988 (Hughes et al 1990) and L-365,260 (Bock et al. 1989), appear to have anxiolytic-like effects in so-called "ethologically valid" rodent models of anxiety such as the rat and mouse elevated-plus maze, the rat social interaction and the mouse light/dark box test (Rataud et al. 1991; Singh et al. 1991). However, as Dooley and Klamt (1993) point out, CCK_B receptor antagonists induce dose-related anxiolytic-like effects only in rodent paradigms that depend upon 'naturally aversive' stimuli to induce anxiogenic-like behaviour. In

conflict or punishment paradigms in which electric shock is used as an anxiogenic stimulus, the effects of CCK_B receptor antagonists are much less robust. For example, using a modified operant punishment procedure in squirrel monkeys, Powell and Barrett (1991) showed that CI-988 (0.03–10.0 mg/kg) had an anxiolytic-like effect, but only at a single dose of 3.0 mg/kg. Singh et al (1991) found that although CI-988 was active over a wide dose-range in the elevated plus maze test (0.01–3.0 mg/kg), it was active at only one dose (0.01 mg/kg) in a shock motivated rat conflict test. Similarly, Dooley and Klamt (1993) report that mice given doses of either 0.0001 or 0.1 mg/kg CI-998 took more shocks than controls in a four-plate test, but intervening doses were without effect. In these studies the anxiolytic-like effect of CI-988 was modest compared to the appropriate positive control group given a benzodiazepine receptor agonist, such as chlordiazepoxide.

There are at least two possible explanations for the lack of a consistent effect of CCK_B receptor antagonists in shock-motivated tests. First, it is possible that the level of fear induced by electric shock is much higher than that induced by the 'natural' aversive stimuli present, for example, in the elevated plus maze test. If this is the case, then CCK_B receptor antagonists might be expected to be effective in tests that induce mild, but not strong, states of fear. This may have clinical implications, as a diagnosis of panic disorder requires that the patient reports the experience of intense anxiety. An alternative explanation is that whilst L-365,260 readily crosses the blood-brain barrier, it is not water soluble and its bioavailability crucially depends on the vehicle in which it is dissolved or suspended (Jackson et al. 1994). Similarly, although CI-988 is water soluble, it crosses the blood-brain barrier poorly (Patel et al. 1994). As a consequence, it may not be possible to achieve brain concentrations of L-365,260 or CI-988 to occupy a sufficient number of central CCK_B receptors to induce a robust anxiolytic-like effect in shock-motivated tests following systemic administration.

In the present study, we sought to evaluate whether either of these explanations, or a combination of them, accounted for the inconsistent effects of CCK_B receptor antagonists in shock motivated tests. We used three rodent screens sensitive to conventional anxiolytic drugs: (i) a rat elevated plus maze test (EPM); (ii) a rat conditioned-suppression-of-drinking (CSD) test; (iii) a rat conditioned-emotional-response (CER) test. In addition, L-365,260 and CI-988 were evaluated in a primate conflict procedure (PC). Finally, in order to address the pharmacokinetic and brain penetration problems of L-365,260 and CI-988, we examined the effects of L-740,093, a recently developed highly selective, water soluble CCK_B receptor antagonist which has improved bioavailability and also readily crosses the blood-brain barrier (Showell et al. 1994).

Materials and methods

Animals

Three to 4 month-old Sprague-Dawley (225–275 g) rats were used in the EPM test. Hooded PVG rats (300–350 g) were used in the CSD and CER tests because pilot studies showed that they maintained a more stable instrumental rate on operant schedules of reinforcement during and between drug tests than Sprague- Dawley rats. In the CSD test the rats were water deprived for 22.5 h in each 24-h period. In the CER test the rats were maintained at 85% of their free-feeding weight by post-sessional feeding. Animals were maintained on a 12/12-h light-dark cycle in humidity and temperature controlled rooms and were obtained from Bantin and Kingman, Hull, UK. Four adult male squirrel monkeys (*Saimiri sciureus*; 800–1200 g), maintained at 85–90% of their free feeding weight appropriate for their age, served as subjects for the PC paradigm.

Drugs

L-740,093 [(3*R*)-*N*-[5-(3-azabicyclo[3.2.2]nonan-3-yl)-2,3-dihydro-1-methyl-2-oxo-1H-1,4-benzodiazepin-3-yl]-*N'*-[3-methylphenyl] urea hydrochloride](Showell et al. 1994), L-365,260 (3*R*)-*N*-[2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl]-*N'*-[3-methylphenyl]urea](Bock et al. 1989) CI-988 ([*R*-(*R**,*R**)]-4- [[2-[[3-(1H-indol-3-yl)-2-methyl-1-oxo-2-[[(tricyclo[3.3.1.1.^{3,7}]dec-2-yloxy) carbonyl]amino]propyl]amino]-1-phenylethyl]amino]-4-oxobutanoate *N*-methyl-D-glucamine)(Hughes et al. 1990) and FG 8205 (7-chloro-5,6-dihydro-5-methyl-6-oxo-3-(5-isopropyl-1,2,4-oxadiazol-3-yl)-4H-imidazol[1,5a][1,4]benzodiazepine)(Tricklebank et al. 1990) were synthesised by the Merck, Sharp and Dohme Neuroscience Research Centre's medicinal chemistry group. Bretazenil was kindly donated by Hoffmann La Roche and diazepam was obtained from Sigma, St Louis, USA. For experiments using rodents, all compounds were prepared freshly each day and injected in a volume of 1 mg/ml. L-740,093 and CI-988 were dissolved in 0.9% saline and L-365,260 was dissolved in labrafil (ALFA Chemicals, Berks, UK.). Bretazenil was dissolved in 100% polyethylene glycol (PEG 400). FG 8205 and diazepam were suspended in 0.5% methyl cellulose + 0.2% Tween 80. All compounds were administered IP 30 min before the beginning of each experiment. For experiments using squirrel monkeys, CI-988 was dissolved in sterile water and administered IM as described by Powell and Barrett (1991). L-365,260 was suspended in 90% Imwitor/10% Tween and diazepam in 0.5% methylcellulose prior to oral administration.

Apparatus and procedures

Elevated plus maze

The EPM was made from black Perspex and its floor was covered with black rubber matting. The maze was arranged in a "+" shape with two open arms facing each other. The other two arms were enclosed by 40 cm high walls. Each arm measured 10 × 50 cm and was raised 50 cm above the floor. Four fluorescent strip-lights were mounted on the ceiling, one above each arm, and illuminated the maze with plane-polarised light. At the beginning of a trial the rat was placed in the centre of the maze with its nose facing one of the open arms and allowed to freely explore for 5 min. The rats were observed via a video camera, fitted with a polarising lens, mounted directly above the centre platform of the maze. The camera was connected to a television monitor and a BBC microcomputer via a VP112 tracking unit (HVS, London) housed in an adjacent room. The walls and ceiling of the maze room were painted in matt black and a black rubber mat completely covered the floor. As a

consequence of the black maze and room, a white rat provided a high contrast image that could be tracked through the maze by the VP112 unit. The computer software calculated the time the animal spent in the open and closed arms, the total distance the rat travelled while in the maze, the distances travelled in the closed and open arms and the number of entries into closed and open arms.

Before testing of the CCK_B receptor antagonists began, the sensitivity of the test was assesed by establishing a dose-response relationship for the low efficacy benzodiazepine receptor partial agonist, bretazenil. Thus, 60 animals were assigned to one of five groups and given either vehicle or 0.3, 1.0, 3.0, 10.0 mg/kg bretazenil. For the L-740,093 experiment, 60 animals were assigned to one of five treatment groups (n = 12) receiving Vehicle; 0.1, 0.3, or 1.0 mg/kg L-740,093 or 3.0 mg/kg diazepam. The effects L-365,260 were evaluated over a wide dose range in two experiments. In the first L-365,260 experiment two doses of CI-988 (0.01 and 0.1 mg/kg) were also evaluated and 108 animals were assigned to nine treatment groups: vehicle; 0.00001, 0.0001, 0.001, 0.01 or 0.1 mg/kg L-365,260 or 0.01 or 0.1 mg/kg CI-988; or 3.0 mg/kg bretazenil. In the second experiment, 60 animals were assigned to five groups (n = 12): vehicle, 1.0, 3.0, or 10.0 mg/kg of L-365,260; or 3.0 mg/kg bretazenil. Each experiment was carried out over 2 days, with six animals from each group tested on each day.

Conditioned-suppression-of-licking

Twelve standard operant boxes were fitted with grid floors through which scrambled electric shock (0.4 mA) could be delivered to the animal's feet. A food magazine was placed 130 mm above the grid floor in the middle of the front wall through which the animals could gain access to a metal drinking spout recessed 5 mm behind the front wall. This arrangement required the animal to stand with its front paws on the wall in order to lick the drinking spout which was connected to a water reservoir via a peristaltic pump. When the rat licked the spout a circuit was made between the tongue, the front wall of the operant chamber and a lickometer connected to an Archimedes A5000 computer running the real time language Arachnid (Paul Fray Ltd. Cambridge). The operation of the peristaltic pump was also controlled by the computer and as a consequence, water (0.1 ml) could be delivered to the spout after a random interval between reinforcers. A houselight (2.4 W) was positioned in the middle of the front wall 250 mm above the grid floor, the illumination of which served as a conditioned stimulus (CS) during conditioning sessions.

On day 1, thirsty rats were placed in the operant chamber for 30 min and licking the metal spout was reinforced with 0.1 ml water according to a random interval (RI) 5 s schedule. This procedure rapidly established licking and on the second, third and fourth successive days the schedule was increased to RI 60 s. On the fifth day, the conditioning day, the session length was increased to 60 min. At three 15 min intervals the houselight was illuminated for 60 s, and 1 s before the houselight was switched off a 0.4-mA shock was delivered to the feet of the animals and terminated at the same time as the light was switched off. Thus, the rats received three light-shock pairings, and as a consequence when the light was presented for the third time their licking-rates were suppressed. The degree of suppression was quantified by expressing it as a ratio: Suppression ratio = CS rate/(CS rate + Pre CS rate), where the "CS rate" is the number of licks during the light presentation and the 'Pre CS rate' is the rate during the minute immediately before the light presentation. Rats with suppression ratios for the third light presentation $>= 0.15$ were excluded from drug testing. Thus only rats that had learnt the light-shock relationship, as indicated by a suppression ratio < 0.15 on the third trial, were tested. On day 6 the rats were given a further 30-min RI 60-s session and on day 7 were rested. On day 8, the test day, the procedure was identical to that on the conditioning day, with the exception that electric shocks were not delivered during the light presentations.

The sensitivity of this screen to benzodiazepine receptor agonists was established with FG 8205. The training criterion was achieved by 53 out of 60 rats which were randomly allocated to five groups (n = 10 or 11) and given either vehicle 0.3, 1.0, 3.0 or 10.0 mg/kg FG 8205. In the L-740,093 experiment, 55 of the 60 animals trained had suppression ratios of less than 0.15 for the final light/shock presentation and they were assigned to one of five treatment groups (n = 11): vehicle; 0.1, 0.3 or 1.0 mg/kg L-740,093 or 10.0 mg/kg FG 8205. In the L-365,260 experiment, 48 of the 60 animals trained met the < 0.15 suppression ratio criterion and they were assigned to six treatment groups (n = 8): vehicle, 0.0001, 0.001, 0.01, or 0.1 mg/kg L-365,260 or 10.0 mg/kg FG 8205. In the CI-988 experiment, 53 animals had ratios < 0.15 by the end of training. They were assigned to six groups (n = 8 or 9): vehicle, 0.01, 0.1, 1.0 or 10.0 mg/kg CI-988 or 10.0 mg/kg FG 8205.

Conditioned emotional response test

Sixty rats were trained in eight standard operant chambers housed in sound and light resistant boxes fitted with ventilating fans. Each chamber was fitted with a retractable lever. The lever was positioned on the front wall 70 mm above the grid floor and 20 mm to the right of a food trough positioned in the middle of the front wall 10 mm above a grid floor. Scrambled electric shock could be delivered to the animal's feet via the grid floor and 45 mg food pellets (Bioserv, Sandown Scientific, Esher, UK) could be delivered into the food trough from a pellet dispenser. A houselight was also positioned in the middle of the front wall 250 mm above the grid floor. On the first 2 days of training rats were placed in the operant chambers for 30 min and food pellets were delivered on average every 60 s. During the next 2 days the rats were placed in the chamber with the lever extended into the box. Each press of the lever delivered a food pellet and the session ended after 30 min or when 30 food pellets had been delivered. During the next two weeks an RI 60 s schedule was introduced and the session length was extended to 60 min. On this schedule, food pellets are available on average every 60 s and were delivered contiguously with a lever press. Initially the RI parameter was set to 7 s and gradually increased (15 s, 30 s), over the 2-week period to 60 s. The animals continued on this schedule for a further 2 weeks, during which lever pressing rates stabilised and did not vary by more than 10% from day to day.

In order to establish tolerance to the lever pressing rate-reducing effects of benzodiazepine receptor agonists, all the rats were given 10.0 mg/kg diazepam 30 min before the beginning of a session for 4 consecutive days. During the first of these sessions lever pressing rates were reduced to approximately 20% of the baseline lever pressing rate, but by the fourth session they had returned to approximately pre-diazepam lever pressing rates. Forty-eight rats with lever pressing rates closest to their pre-diazepam rates were selected from the 60 trained rats and served as subjects for the drug-testing sessions. Following the induction of tolerance to the rate-decreasing effects of diazepam, conditioned-suppression training began. Once between minutes 15 and 25, and minutes 35 and 45 into the session the houselight was switched on for 60 s, and 1 s before it was switched off a 0.4-mA scrambled electric shock was delivered to the rat's feet. The electric shock and the light then terminated together. During the 'light-on' period food pellets were delivered as normal. This procedure readily established conditioned suppression of lever pressing during the period when the light was on. Suppression ratios were calculated as described for the CSD procedure using the lever pressing rates 1 min immediately before and during illumination of the light (CS).

When conditioned suppression had been established (normally no more than two sessions were required), the probability of a shock occurring after a light presentation was reduced from 1.0 to 0.1, a level that, in general, maintained suppression ratios at less than 0.15. The day before each test day, the rats with suppression ratios less than 0.15 were randomly assigned to their respective drug groups and an analysis of variance was performed on the suppression ratios and mean lever pressing rates to ensure that there were no a priori significant differences between the drug groups.

The sensitivity of the test to benzodiazepine receptor agonists was established using bretazenil. The training criteria was met by 43 of the 48 rats trained and they were assigned to five groups (n = 8 or 9): vehicle, 0.1, 0.3, 1.0 or 3.0 mg/kg of bretazenil. In the L-740,093 experiment, 39 animals met the criterion and were assigned to one of five treatment groups (n = 7 or 8): vehicle, 0.1, 0.3, or 1.0 mg/kg L-740,093 or 3.0 mg/kg diazepam. The effects of L-365,260 were assessed in two experiments: in the first, 48 animals were assigned to five treatment groups (n = 8): vehicle, 0.01, 0.1 or 1.0 mg/kg L-365,260 or 3.0 mg/kg bretazenil; in the second 39 animals were assigned to five treatment groups (n = 7 or 8): vehicle, 0.3, 1.0 or 3.0 mg/kg L-365,260 or 3.0 mg/kg bretazenil. The effects of CI-988 were also evaluated in two experiments: In the first, 40 animals were assigned to five groups (n = 8): vehicle, 0.1, 0.3, or 1.0 mg/kg CI-988 or 3.0 mg/kg bretazenil; In the second, 42 animals were assigned to five groups (n = 8 or 9): vehicle, 1.0, 3.0 or 10.0 mg/kg CI-988 or 3.0 mg/kg bretazenil.

Squirrel monkey conflict procedure

Each monkey was restrained at the waist in a Perspex chair and placed in an operant box controlled by a BBC Master Computer running the real time control language, Spider (Paul Fray Ltd. Cambridge). The monkey faced a retractable lever, a red and a white stimulus lamp and a food hopper into which banana flavoured rewards (Bioserv, Sandown Scientific, Esher, UK) were delivered. The distal portion of the monkey's shaved tail was held in a Perspex stock and electric shock delivered via two brass electrodes resting on the tail (370 V (peak) AC, 100 Hz, 500 ms, 0.1–5 mA).

The paradigm was based on that described by Weissman et al. (1984). Each session comprised five alternating cycles of unpunished and punished responding. Unpunished components were signalled by the illumination of the white lamp. During these components every 30th lever press resulted in the delivery of a banana pellet (FR30). After 3 min had elapsed the white light was extinguished and a 30-s timeout period commenced during which lever pressing was not rewarded. At the end of this time the red lamp was illuminated and every 30th lever press now resulted in the simultaneous delivery of a food pellet accompanied by a brief electric shock to the tail. The shock intensity was titrated for each individual monkey to the minimum level necessary to maintain consistent suppression of lever pressing. Animals were able completely to avoid electric shocks by withholding lever presses when the red lamp was illuminated. After 3 min the red light was extinguished and a further 30-s timeout ensued before the cycle was repeated.

Monkeys were dosed once per week with test compounds, diazepam (0.1, 0.3 and 1.0 mg/kg, PO), CI-988 (1.0, 3.0 and 10.0 mg/kg) or L-365,260 (1.0, 10.0 and 50.0 mg/kg), 60 min prior to behavioural testing and the total number of responses made during punished and unpunished cycles were recorded. Each monkey received every treatment according to a quasi-Latin square design.

Statistical analysis

The BMDP programs 7D and 2V provided one- and two-way analyses of variance respectively. Post hoc Newman Keuls tests were used to determine group differences ($P < 0.05$).

Results

Elevated plus maze

Figure 1 shows the mean time spent on the open arms of the maze (expressed as a percentage of the total time in the maze, %TIME) and the mean number of open arm entries (ENT) for bretazenil (a), L-740,093 (b), L-365,260 (c) and L-365,260 and CI-988 (d). All the doses of bretazenil administered significantly increased the time the animals spent on the open arms of the maze [$F(4, 54) = 8.49$, $P < 0.0001$]. Similarly, all but

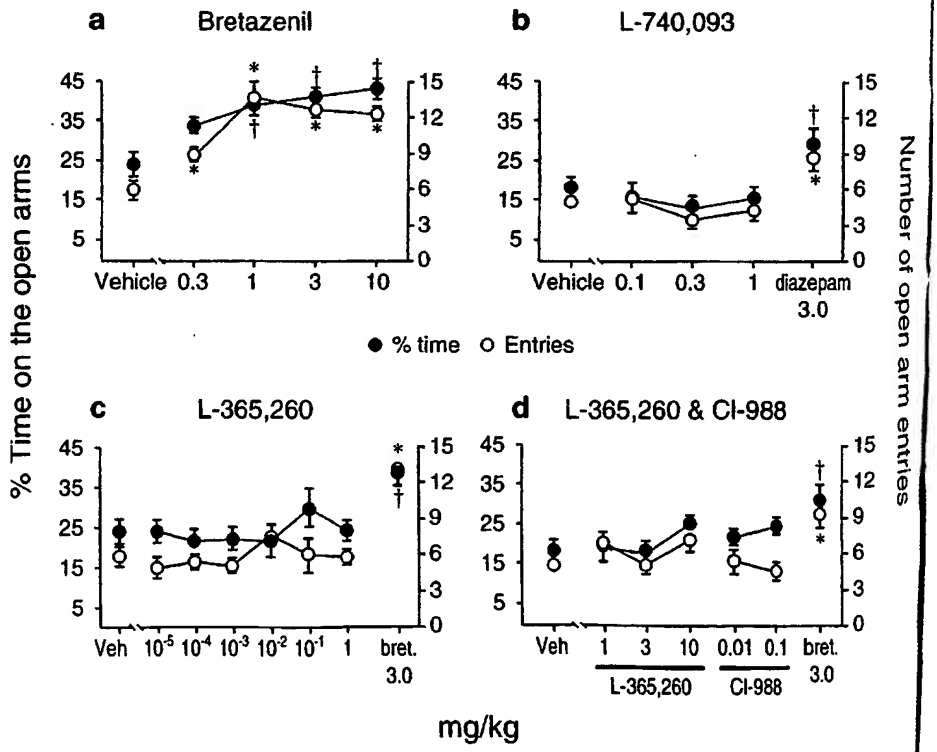

Fig. 1 Effect of CCK_B receptor antagonists in the elevated plus maze test. Results show the mean time spent on the open arms, expressed as a percentage of the total time in the maze and the mean number of open arm entries for each treatment group (± SEM, n=11–12) following a 30 min pretreatment (IP) of vehicle or compound. * Indicates a significant difference in the time spent on the open arms, and a † a significant difference in the number of arm entries compared to the vehicle-treated control group (Newman-Keuls tests, $P < 0.05$)

the 0.3 mg/kg dose of bretazenil significantly increased the number of entries onto the open arms of the maze. None of the doses of L-740,093, L-365,260, or CI-988 administered increased the mean time spent on the open arms or increased the mean number of entries to the open arms. In contrast, the positive control groups given diazepam (L-740,093 experiment) or bretazenil (L-365,260 experiments) spent significantly more time in, and made more entries to, the open arms when compared to the vehicle-treated control group (L-740,093; %TIME: treatment [$F(4, 53) = 3.89$, $P < 0.01$)]; ENT: treatment [$F(4, 53) = 5.33$, $P < 0.01$]). In the first L-365,260 experiment (0.0001–1.0 mg/kg L-365,260,) there was no significant effect of day [$F(1, 71) = 1.46$, $P = 0.23$] nor a treatment × day interaction [$F(5, 71) = 1.92$, $P = 0.10$] and as a consequence the data were collapsed across the day factor and analysed on treatment only: %TIME: [$F(7, 84) = 3.47$, $P < 0.01$)]; ENT: [$F(7, 84) = 8.94$, $P < 0.01$]. In the second L-365,260 experiment (1.0–10.0 mg/kg L-365,260; 0.01 and 1.0 mg/kg CI-988) two animals in the vehicle group and one from the 10.0 mg/kg L-365,260 group fell from the maze during testing and were excluded from the data analysis; %TIME: [$F(6, 74) = 5.47$, $P < 0.01$]; ENT: [$F(6, 74) = 6.75$, $P < 0.01$].

Conditioned suppression of drinking

Figure 2 illustrates the effects of FG 8205 (a), L- 740,093 (b), L-365,260 (c) and CI-988 (d) on mean suppression ratios and on mean Pre-CS and CS lick rates. FG 8205 dose-dependently increased suppression ratios [$F(4, 47) = 19.46$, $P < 0.001$] with doses of 3.0 and 10.0 mg/kg FG 8205 significantly increasing suppression ratios above those of the vehicle-treated controls. In the L-740,093, L-365,260 and CI-988 experiment, analyses of variance revealed main effects of treatment, [$F(4, 50) = 8.62$, $P < 0.001$], [$F(5, 42) = 19.34$, $P < 0.001$] and [$F(5, 47) = 8.66$, $P < 0.001$], respectively. Although FG 8205 induced a significant increase in mean suppression ratios compared to the vehicle-treated control group in all three experiments, none of the doses of L-740,093, L-365,260 or CI-988 administered had any significant effect on suppression ratios. None of the CCK_B receptor antagonists administered affected Pre-CS lick rates (analyses of variance by treatment: L-740,093, [$F(4, 50) = 1.40$, $P = 0.25$]; L-365,260, [$F(5, 42) = 0.13$, $P = 0.36$]; CI-988, [$F(5,47) = 1.26$, $P = 0.30$]), indicating that the compounds did not exert non-specific behavioural effects.

Conditioned emotional response

Figure 3 shows the effects of bretazenil (a), L-740,093 (b), L-365,260 (c) and (d) and CI-988 (e) and (f) on mean suppression ratios and on mean Pre-CS and CS lever pressing rates. Bretazenil had a dose-proportional effect [$F(4, 38) = 3.87$, $P < 0.01$], with doses of 0.3, 1.0 and 3.0 mg/kg significantly increasing suppression ratios compared to vehicle-treated control rats. In the L-740,093 experiment none of the doses administered significantly increased mean suppression ratios, whereas diazepam induced a small, but significant increase in the mean suppression ratio when compared to the vehicle-treated control group (one-way analysis for treatment: [$F(4, 34) = 3.43$, $P < 0.02$]). The effect of

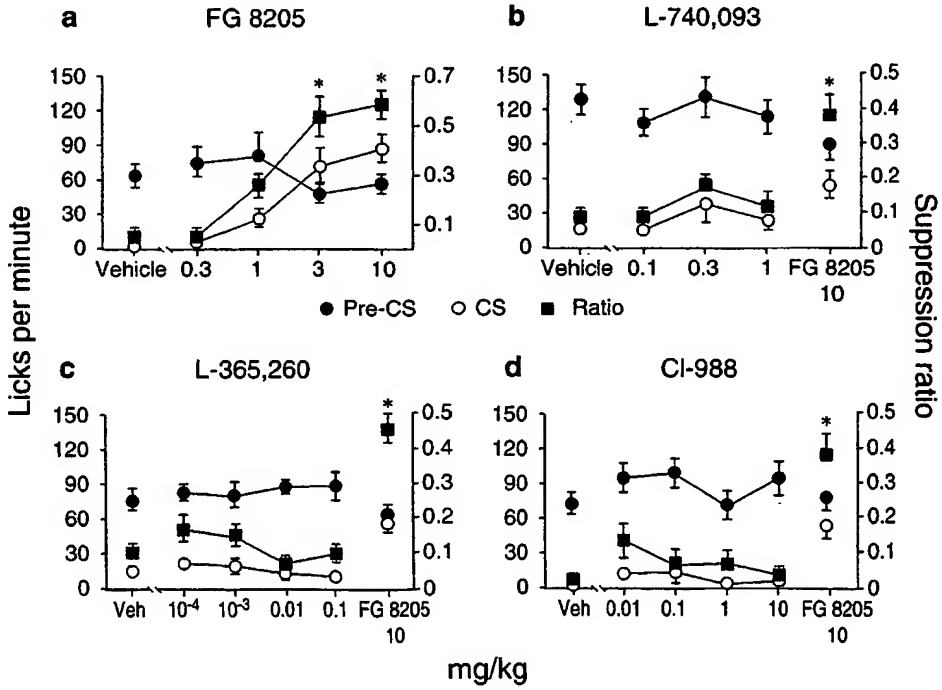

Fig. 2 Effect of CCK_B receptor antagonists in the conditioned suppression of drinking test. The results show the mean number of licks (± SEM, $n = 8$–10) 1 min before (*Pre CS*) and 1 min during (*CS*) the illumination of a light that 48 h earlier predicted the delivery of a mild electric shock, and the suppression ratio for each treatment group. * indicates a significant difference in the suppression ratio compared to the vehicle-treated control group (Newman-Keuls tests, $P < 0.05$)

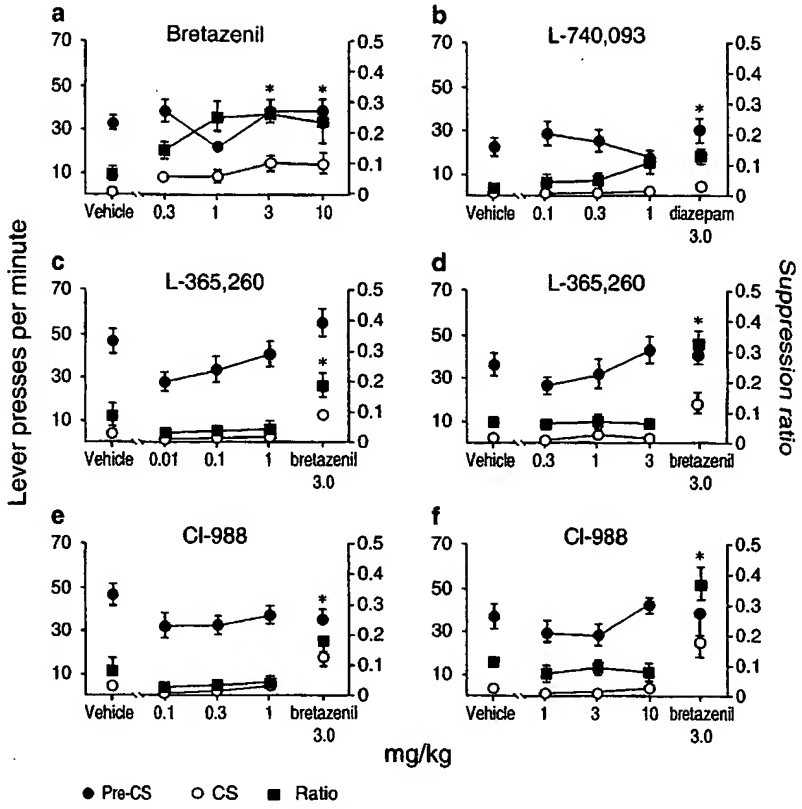

Fig. 3 Effect of CCK_B receptor antagonists in the conditioned emotional response test. The mean number of lever presses (± SEM, n = 8–10) 1 min before (*Pre CS*), and 1 min during (*CS*), the illumination of a light that predicted with a probability of 0.1 the delivery of a mild electric shock and the suppression ratio, are shown for each treatment group (Diazepam and L-365,260, PO; CI-988, IM). * Indicates a significant difference in the suppression ratio compared to the vehicle-treated control group (Newman-Keuls tests, $P < 0.05$)

L-365,260 was evaluated in two experiments. In the first experiment, L-365,260 was without effect at all the doses tested (0.01–1.0 mg/kg), although bretazenil did increase the mean suppression ratio compared to the vehicle-treated control group (one way analysis for treatment: [$F(4, 43)=5.05$, $P < 0.002$]). In the second L-365,260 experiment bretazenil again significantly increased the mean suppression ratio compared to the vehicle-treated control group, but the doses of L-365,260 (0.3–3.0 mg/kg) given again had no significant effects on suppression ratios (one-way analysis for treatment: [$F(4, 34) = 11.83$, $P < 0.001$]).

The effect of CI-988 on conditioned suppression was also evaluated in two experiments. In the first, bretazenil significantly increased mean suppression ratios compared to the vehicle-treated control group, but CI-988 (0.1–1.0 mg/kg) was without effect (one-way analysis for treatment: [$F(4, 35) = 13.52$, $P < 0.001$]). In the second experiment a similar pattern of results were observed. Bretazenil again significantly increased the mean suppression ratio compared to the vehicle-treated control group and CI-988 (1.0–10.0 mg/kg) was again without effect (one-way analysis for treatment: [$F(4, 37) = 13.72$, $P < 0.001$]).

Primate Conflict Procedure

On control days when monkeys received no drug treatment, the number of lever presses made during punished cycles was approximately 5% of that during unpunished cycles. Administration of diazepam (0.1–1.0 mg/kg) caused a dose-proportional increase in punished responding up to 70% of unpunished control levels (one-way analysis for treatment: [$F(4, 12) = 6.67$, $P < 0.05$]). In contrast, lever pressing during unpunished cycles was not altered by treatment with diazepam in this dose range [one-way analysis for treatment: $F(4, 12) = 0.77$, $P > 0.05$; Fig. 4a]. Unlike the release of punished lever pressing observed after treatment with diazepam, administration of CI-988 (1.0–10.0 mg/kg) or L-365,260 (1.0–50.0 mg/kg) did not alter lever pressing during either punished [$F(4, 12)$ 0.48, $P > 0.05$] or unpunished [$F(4, 12)$ 0.99, $P > 0.05$] components (Fig. 4b and c).

Discussion

L-740,093 is a highly selective, water soluble CCK_B receptor antagonist which has high bioavailability and readily crosses the blood-brain barrier (Showell et al. 1994). However, in the present study, L-740,093 (0.1–1.0 mg/kg) failed to induce an anxiolytic-like effect in either ethological (EPM) or classical operant (CSD and CER) animal screens for anxiolytic agents. It has been reported previously that CI-988 has dose-proportional anxiolytic-like effects in ethological paradigms (Rataud et al. 1991; Singh et al. 1991) and non-dose-proportional effects in paradigms in which

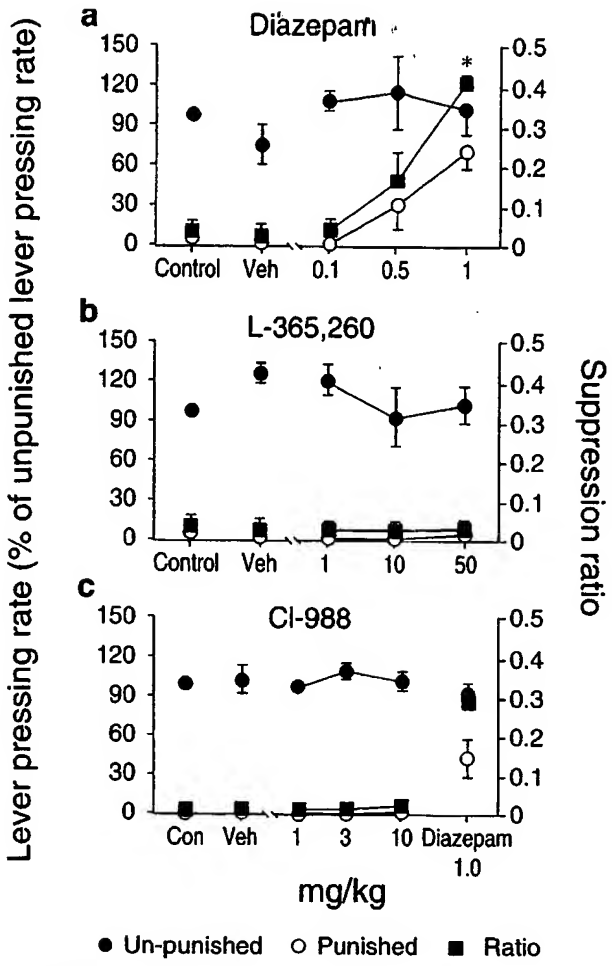

Fig. 4 Effect of CCK_B receptor antagonists in the primate punishment test. The mean number of lever presses (± SEM) expressed as a percentage of the control (non-injected) unpunished lever pressing rate during unpunished and punished components and the suppression ratio, are shown for each treatment group. * Indicates a significant difference in the suppression ratio compared to the vehicle-treated control group (Newman-Keuls tests, $P < 0.05$ assumed)

electric shock is used as a reinforcer (Powell and Barrett 1991: Dooley and Klamt, 1993), but there was no evidence of a similar pattern of results in the present experiments. The CCK_B receptor antagonists CI-988 and L-365,260 were also without effect in the animal tests described above and in a primate anxiolytic screen.

The efficacy of anxiolytic agents in a given animal behavioural test is likely to vary according to the level of fear or anxiety induced by the aversive stimulus used. This is illustrated by the varied activities of compounds used in the present study across a range of anxiolytic paradigms. Thus, with the full benzodiazepine receptor agonist, diazepam, and partial receptor agonist, FG 8205, similar doses of each of the compounds were active in all of the rodent tests here described, whereas the partial receptor agonist, bretazenil, which has lower intrinsic activity at the GABA-A/benzodiazepine receptor than FG 8205 (Tricklebank et al. 1990), was active only in the elevated plus maze and CER tests. This phenomenon is similarly illustrated in the report by Nevins and Anthony (1994), who showed that a number of $5\text{-}HT_3$ receptor antagonists were active in a conditioned fear paradigm only when low intensity shock was used, whereas diazepam was active regardless of shock intensity.

Unlike bretazenil, however, the CCK_B receptor antagonist, L-365,260, did not exhibit significant anxiolytic-like activity in the CSD, CER or elevated plus maze tests. One interpretation of this finding is that the anxiolytic efficacy of CCK_B receptor antagonists may be very low. Clearly, this may be for reasons other than a lack of involvement of the CCK_B receptor in modulating behavioural responses to novelty and fear. Thus, although an antagonist of high affinity and selectivity for the CCK_B receptor in vitro (Chang and Lotti 1986), L-365,260 is very poorly water soluble and, despite being readily able to penetrate the CNS, the failure to see behavioural effects with the compound could reflect the use of a formulation (labrafil) from which the compound may have precipitated on IP injection in a poorly absorbable form. However, pharmacokinetic studies suggest that this is unlikely (Hargreaves and Lin 1992). Moreover, this question was obviated in the present experiments by the use of the water soluble and brain penetrating CCK_B receptor antagonist, L-740,093, at doses that are sufficient to occupy more than 90% of brain CCK_B receptors for the duration of the behavioural assays (Showell et al. 1994; M. Graham, personal communication): this compound was also inactive in all of the rodent anxiolytic tests. CI-988 was similarly without effect, although this may simply reflect the poor ability of the compound to penetrate the CNS (Patel et al. 1994). On this basis, there is clearly no evidence that CCK_B receptor antagonists have any significant activity in conventional anxiolytic screens sensitive to benzodiazepines, regardless of whether they are ethological in nature or derived from the principles of behavioural conditioning.

Nevertheless, it has been previously reported that CI-988 and L-365,260 have dose-proportional effects in ethological animal models of anxiety (Rataud et al. 1991; Singh et al. 1991) and non-dose-proportional effects in animal models in which electric shock is used as a reinforcer (Powell and Barrett, 1991; Singh et al 1991; Dooley and Klamt, 1993). The lack of effect of CCK_B receptor antagonists in the present experiments is not, therefore, readily explained. It is possible that methodological differences may account for the presence, in previous study, and the absence, in the present studies, of an anxiolytic-like effect by CCK_B receptor antagonists. The rodent shock-motivated tests used in the present study employed conditioned emotional response procedures rather than the conditioned punishment procedure described by Singh et al. (1991). Thus, in the present study response suppression was induced by a signal for response-independent shock, whereas the signal in the Singh et al experiment

indicated a period during which responses would be punished (response contingent shock). However, in conditioned punishment procedures a signal for response contingent shock does not usually induce anxiety-like behaviour in well-trained animals, because the delivery of shock is avoidable (Kamin et al. 1963). As "response disinhibition" is one of the behavioural effects of benzodiazepines (Gray 1982), it is possible that the increase in punished responding observed in punished paradigms might be a reflection of response disinhibition rather than a reduction in the magnitude of the fear response during the punished component. If this were the case then it might be expected that CCK_B receptor antagonists would also disinhibit responses in operant procedures such as the differential reinforcement of low rates of responding schedule (DRL) in which the withholding of a response for longer than a specified period (response inhibition) is required for reinforcement. However, neither CI-988 nor L-365,260 had such an effect in a DRL 20s schedule, whereas bretazenil significantly reduced the animals' ability to withhold a response (Dawson et al. unpublished data).

The magnitude of the anxiolytic-like effect of CI-988 in the punished procedure described by Singh et al. (1991) was small (mean increase in punished responding ~ 25%) compared to the effect of chlordiazepoxide (~137%) and occurred only at one dose (0.01 mg/kg). In the Dooley and Klamt (1993) experiment the magnitude of the effect of CI-988 in the mouse four-plate test was again small compared to diazepam, and not dose dependent. Furthermore, between 20 and 40 animals were required at each dose in order to achieve statistical significance. Taken together, these results, and the failure to see anxiolytic-like effects of CCK_B receptor antagonists in the present rodent shock-motivated experiments, suggests that the anxiolytic-like effects of CCK_B receptor antagonists are slight and may only be observed in very limited circumstances. Moreover, the lack of effect of L-365,260 and CI-988 in the present primate conflicit procedure was not surprising, given that their effects in the Powell and Barrett (1991) experiment were confined to a narrow dose range and again the effect was not dose proportional. Furthermore, of the five monkeys included in the Powell and Barret experiment, two were tested with alternating punished/unpunished components, whereas the remaining three were tested with one unpunished and one punished component. Since the animals were tested under different conditions, it would not have been valid statistically to analyse the combined data. Consequently, combination of the data from the two groups of animals for illustrative purposes can give no statistical support to the small increase in punished responding at one dose being meaningful. It is worth noting, however, that in the primate punishment procedure used in the present experiments, a fixed ratio schedule maintained lever pressing, whereas a fixed interval schedule maintained lever pressing in the Powell and Barrett experiment. As drug-induced changes in lever pressing rates can vary depending on schedule maintaining the lever pressing rate, Dews and De Weese (1977) it may be that fixed interval schedules are more sensitive to the rate increasing effects of CCK_B antagonists than fixed ratio schedules. This differential sensitivity may account for the small increase in punished responding seen in the Powell and Barrett experiment.

Although CCK_B receptor antagonists appear to have anxiolytic-like effects in certain ethological screens, the effective dose range varies markedly between paradigms. Thus, for CI-988 active dose ranges vary from 1.0 µg/kg in the mouse light/dark box and 0.01 µg/kg in the marmoset threat test (Costall et al. 1991), to 0.1 mg/kg in the mouse the light/dark box (Singh et al. 1991). In contrast, Hendrie et al. (1993) reported that L-365,260 was without effect in this test. In the elevated plus maze the anxiolytic-like effects of CCK_B receptor antagonists are also inconsistent. Singh et al. (1991) reported that in the rat elevated plus maze the minimum dose of CI-988 that significantly increased the time on the open arms and the number of open arm entries was 0.01 mg/kg (PO). In the mouse elevated plus maze, Rataud et al. (1991) reported that 0.01 mg/kg (IP) of L-365,260 increased the time spent on the open arms and 0.1 mg/kg (IP) significantly increased the number of open arm entries. By contrast, Harro and Vasar (1991) found no effect of L-365,260 in the rat elevated plus, which is in agreement with the lack of effect of L-365,260 observed in the present study.

Thus, even in ethologically based anxiolytic tests, CCK_B receptor antagonists have inconsistent effects which vary markedly in terms of both potency and efficacy between laboratories. Inconsistent effects have also been seen in our own laboratory: on one occasion an anxiolytic-like effect of L-365,260 in the CSD test was detected and reported (Dourish et al. 1991) but as shown in the present work, these findings could not be reliably replicated. One conclusion from the overall results might be that the CCK_B receptor does not have a robust influence on fear-motivated behaviour in rodents or subhuman primates. Such a conclusion is in marked contrast to the induction of panic attacks by the rapid intravenous infusion of CCK_B receptor agonists in man and in some species of primate (De Montigny, 1989; Bradwejn et al. 1990; Palmour et al. 1992), but is consistent with the failure of the rapid intravenous or intracerebral infusion of CCK_4 to disrupt the operant response rate of freely moving rats trained to press a lever for food rewards, a procedure sensitive to the benzodiazepine receptor inverse agonist, FG 7142 (Bayley and Dawson, 1993). Similarly intravenous administration of pentagastrin failed to induce the behavioural or cardiovascular disturbances previously reported for β–carbolines in rhesus monkeys (Rupniak et al. 1993).

Thus, animal behavioural tests of anxiety do not give much impetus to the use of CCK_B receptor antagonists for the treatment of human panic and/or anxiety disorders where the intensity of anxious behaviour must be great compared to that induced by exposing, for example, an animal to the open arms of an elevated plus maze. It remains to be seen whether the ability of the acute administration of L-365,260 to block CCK_B induced panic in man (Traub et al. 1993) is a good indicator of the therapeutic potential of CCK_B receptor antagonists in panic disorder. Although preliminary findings with repeated dosing of L-365,260 are not encouraging (Kramer et al. 1994), the formulation difficulties associated with the compound prevented a definitive evaluation of the therapeutic utility of CCK_B receptor antagonists. A more rigorous appraisal of the anxiolytic efficacy of these agents in man is awaited, using more suitable compounds. The outcome of such studies may help to clarify the predictive validity of the wide range of anxiolytic screens currently employed in animals.

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